

**The effects of stearidonic acid on 3T3-L1 adipocytes**

by

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## Abstract

The n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in fish oil have been reported to have anti-obesity, anti-inflammation, and insulin-sensitizing effects. The botanical fatty acid, stearidonic acid (SDA), has been targeted as a potential biologically active surrogate for EPA. The aim of this study was to investigate whether SDA has potential anti-obesity, anti-inflammatory and insulin-sensitizing properties using the 3T3-L1 adipocyte cell culture model. In comparison to an ethanol control, SDA inhibited 3T3-L1 adipocyte differentiation by significantly decreasing (49%;  $P < 0.05$ ) triglyceride accumulation, significantly decreasing (45-73%;  $P < 0.05$ ) adipogenic transcription factor gene expression (C/EBP $\alpha$ , C/EBP $\beta$  and PPAR $\gamma$ ), and significantly decreasing (59-96%;  $P < 0.05$ ) expression of genes involved in lipid accumulation (aP2, FAS, GLUT4, LPL and SCD-1). Furthermore, SDA significantly decreased (87%;  $P < 0.05$ )  $^3\text{H}$ -thymidine incorporation during the second cycle of 3T3-L1 cell division via cell proliferation assay. To evaluate the effect of SDA on inflammation, gene expression of the inflammatory cytokine IL-6 was measured in 3T3-L1 adipocytes exposed to macrophage-derived conditioned medium pretreated with or without SDA for 24 h. Compared to BSA control, SDA significantly decreased IL-6 expression by 63% ( $P < 0.05$ ). EPA and ALA decreased IL-6 expression by 41% and 39%, respectively ( $P < 0.05$ ). To determine the effects of SDA on insulin sensitivity, insulin-stimulated glucose uptake assays were performed on 3T3-L1 adipocytes incubated in the absence or presence of SDA

(24 h). SDA significantly increased insulin-stimulated glucose uptake by approximately 40% ( $P < 0.05$ ). In addition, Western Blot analysis revealed that SDA treatment had no significant effects on insulin signaling through phosphorylation of Akt. The effect of SDA on PPAR $\gamma$  activity in mature adipocytes was measured by luciferase assay in HEK293 cells. SDA significantly increased transcription activity 1.8 fold ( $P < 0.05$ ). EPA and DHA significantly activated the transcriptional reporter 2.6 and 2.7 fold, respectively ( $P < 0.05$ ). We conclude that SDA exhibited effects on inhibiting adipocyte differentiation, attenuating inflammation, and improving insulin sensitivity in 3T3-L1 adipocytes. Thus, SDA could be a botanical source of n-3 PUFA in the treatment of obesity and type 2 diabetes.

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## List of Abbreviations

AA	arachidonic acid	DHA	docosahexaenoic acid
AdipoR	adiponectin receptor	DM	differentiation medium
AHA	American Heart Association	DMEM	Dulbecco's Modified Eagle's Medium
ALA	alpha-linolenic acid	DNA-PK	DNA-activated protein kinase
AMPK	AMP-activated protein kinase	EPA	eicosapentaenoic acid
aP2	adipocyte fatty acid binding protein	eIF2	Eukaryotic translation initiation factor 2
BMI	body mass index	ELISA	Enzyme-linked immunosorbent assay
BSA	bovine serum albumin	FCS	fetal calf serum
CAP	C-Cbl-associated protein	FBS	fetal bovine serum
C/EBP	CCAAT/enhancer-binding protein	FFA	free fatty acid
COX-2	cyclooxygenase-2	GLA	gamma-linolenic acid
CRP	C-reactive protein	GLUT4	glucose transporter 4
CVD	cardiovascular disease	GSK3-β	Glycogen synthase kinase 3 beta
2-DOG	2-deoxyglucose	HDL	high-density lipoprotein
DAG	diacylglycerol		
DEX	dexamethasone		



HEK 293	Human Embryonic Kidney 293	PH	pleckstrin homology
IBMX	isobutylmethylxanthine	PI3	phosphatidylinositol 3
IKK	inhibitor kappaB kinase	PKC	protein kinase C
IL	interleukin	PPAR	peroxisome proliferator- activated receptor
iNOS	inducible nitric oxide synthase	PPRE	PPAR response elements
IRS	insulin receptor substrate	PTB	phosphotyrosine binding
JNK	Jun N-terminal kinase	PUFA	polyunsaturated fatty acid
LA	linoleic acid	RXR	retinoid X receptor
Lo-15-1	15-lipoxygenase-1	RT-PCR	quantitative real-time reverse transcription-polymerase chain reaction
LPL	lipoprotein lipase	WHO	World Health Organization
LPS	lipopolysaccharide	VLDL	very-low-density lipoprotein
MAPK	mitogen-activated protein kinase	SCD-1	stearoyl-CoA desaturase 1
MCE	mitotic clonal expansion	SREBP-1	Sterol regulatory element binding protein-1
MCP-1	monocyte chemotactic protein 1	SDA	stearidonic acid
NFDM	non-fat dry milk	SDS-	sodium dodecyl sulfate
OA	oleic acid	PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline	SOCS	suppressors of cytokine signalling
PDK1	phosphoinositide-dependent kinase 1		
PEPCK	phosphoenol pyruvate carboxykinase		
PGE2	prostaglandin E2		

SoHo	sorbin homology
SOS	son-of-sevenless
TBS	tris buffered saline
TG	triacylglycerol
TLR	toll like receptor
TNF	tumor necrosis factor
TZDs	thiazolidinediones

## **Chapter 1 Introduction**

In recent years, obesity has become a serious health problem on a global scale. The World Health Organization (WHO) estimates that there are over 400 million obese adults around the world, with another 1.6 billion overweight. Furthermore, WHO projects that by 2015, approximately 2.3 billion adults will be overweight, and more than 700 million will be obese (3). Overweight and obesity are closely linked to various diseases such as type 2 diabetes, hyperlipidemia, hypertension, coronary heart disease, and cancer (4). Research on how to prevent and treat obesity and related diseases is an area of great interest. Genetic and environmental factors, including exposure to various nutrients and dietary behaviors, can influence the development of obesity. Dietary Fish oil containing long chain n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) has been shown to protect against the development of obesity and reduce body fat (5-8). Cold water fish and fish oils are the most direct source of EPA and DHA. However, many individuals cannot tolerate the taste or smell of fish oils, even when provided in capsules. In addition, yields from global fisheries have been reported to be stagnant or declining (9), and there is increasing alarm over levels of methyl mercury in some species of long-lived fish. Hence, there is a need and desire to identify and develop alternative sources of n-3 long chain PUFA.

$\alpha$ -linolenic acid (ALA; 18:3 n-3) is the main n-3 fatty acid available in vegetable oils. However, there is poor conversion of ingested ALA to EPA and DHA because of limited  $\Delta 6$  desaturase activity. Stearidonic acid (SDA; 18:4 n-3) has been targeted as a potential biologically active surrogate for EPA in fish oil due to its relatively efficient conversion following consumption. SDA represents the  $\Delta 6$  desaturation product of ALA, and thus bypasses the rate-limiting step in the conversion of dietary ALA to EPA. When SDA enters the metabolic pathway after the  $\Delta 6$  desaturase step, it is rapidly converted to EPA (10). SDA is found in the seeds and leaves of several plants, including those of the boraginaceae family, such as *Echium plantagineum*, borage, evening primrose and blackcurrent (11). Consumption of SDA as ethyl esters, Echium Oil, or SDA-soybean oil increased EPA levels in plasma, neutrophil, heart, and erythrocyte phospholipids (11-16). A clinical study indicated SDA supplied as ethyl esters is one third as effective as EPA at raising EPA levels in red blood cell and in plasma phospholipids (12). Experimental and clinical studies indicated that SDA shares many of the biological effects of n-3 LCPUFA and functions similarly to dietary EPA. SDA in triacylglycerol can mimic EPA in reducing inflammatory cytokines tumor necrosis factor (TNF) $\alpha$  and prostaglandin E2 (PGE2) release from stimulated whole blood and splenocytes in mice (17). In a rodent model of colorectal cancer, SDA as ethyl esters reduced tumor multiplicity by ~50%, and this effect was as effective as EPA (18). A clinical study has found that SDA and EPA as ethyl esters are more effective than ALA on increasing the omega-3 index, which correlates with risk for a variety of cardiovascular disease endpoints (16). In addition, Echium oil containing 12.5 % SDA has been shown to lower serum triacylglycerols in hypertriglyceridemic humans (11).

Obesity arises from the imbalance between energy intake and energy expenditure. At the cellular level, obesity was originally considered as a disease resulting from an increase in fat cell

number or the size of individual adipocytes. The size of adipocytes increases because of increased storage of triacylglycerols from dietary sources or endogenous lipogenic pathways. The number of adipocyte increases as a result of increased proliferation and differentiation (19). The entire adipogenic process consists of preadipocyte proliferation and their differentiation into mature adipocytes, and this proliferation has proven to be a prerequisite for adipogenesis. The differentiation of adipocytes from preadipocytes has been extensively studied in preadipocyte cell lines such as 3T3-L1 and 3T3-F422A cells. Upon receiving the appropriate combination of adipogenic signals, growth-arrested confluent 3T3-L1 preadipocytes initiate mitotic clonal expansion (MCE) and re-enter the cell cycle for an additional two rounds of division. MCE is a prerequisite for terminal differentiation (20). The peroxisome proliferator-activated receptor (PPAR)  $\gamma$  and CCAAT/enhancer-binding protein (C/EBP) play essential roles in adipogenic differentiation. These two proteins are involved in the sequential expression of adipocyte-specific proteins such as lipoprotein lipase (LPL), adipocyte fatty acid binding protein (aP2) and glucose transporter 4 (GLUT4), which mediate fatty acid or glucose uptake into adipocytes, triglyceride hydrolysis and lipogenesis (21, 22), thereby leading to the characteristic phenotype of mature adipocytes. N-3 PUFA EPA (23) and DHA (24) have been reported to reduce transcription of the differentiation marker genes and inhibit adipocyte differentiation.

There is growing evidence that obesity, insulin resistance and type 2 diabetes are tightly associated with a chronic, low-grade inflammatory state. The inflammatory state associated with obesity appears to be triggered and to reside predominantly in adipose tissue. Adipose tissue plays an important role as an endocrine organ secreting different hormones and cytokines that can augment or impair whole-body insulin sensitivity. Adipose tissue in obese individuals shows increased expression of pro-inflammatory proteins including Tumor necrosis factor-alpha (TNF-

$\alpha$ ), interleukin (IL-6), monocyte chemotactic protein 1(MCP-1), and inducible nitric oxide synthase (iNOS) (25-27). Adiponectin is an adipose-specific plasma protein, which has an anti-inflammatory action (28). Plasma adiponectin concentrations are decreased in obesity and are positively associated with whole-body insulin sensitivity (28-30). Locally secreted chemokines like MCP-1 attract pro-inflammatory macrophages into the adipose tissue where they function to scavenge moribund adipocytes. These tissue macrophages are the main source of pro-inflammatory cytokines that further activate the inflammatory program in neighboring adipocytes. Those secreted pro-inflammatory cytokines cause insulin resistance in adipocytes through activation of several inflammatory pathways such as inhibitor kappaB kinase (IKK) and Jun N-terminal kinase (JNK) that interfere with insulin signaling via serine phosphorylation and subsequent inactivation of insulin receptor substrate (IRS)-1 (31).

Free fatty acids have emerged as a major link between obesity and insulin resistance and type 2 diabetes. Fasting whole-body FFA and glycerol release from adipocytes is increased in obese women compared with lean women (32). Physiological elevations of plasma FFA inhibit acute as well as chronic insulin-stimulated glucose uptake in a dose dependent fashion (32). FFA can activate at least three inflammation-associated proteins including protein kinase C (PKC) delta, IKK and JNK that are involved in the cross-talk between inflammatory and insulin signaling pathways. Insulin sensitivity in animals can be influenced by the manipulation of the dietary fatty acid profile. Diets high in saturated fats or rich in linoleic acid and n-6 fatty acids lead to insulin resistance (33, 34). However, replacement of some of the fat with n-3 LCPUFAs prevents the development of insulin resistance caused by high-fat feeding (33, 34).

The overall objective of our study was to determine whether SDA has potential anti-obesity and insulin-sensitizing properties. We investigated the effects of SDA on the

proliferation and differentiation of 3T3-L1 preadipocytes. We also examined the effect of SDA on macrophage-induced inflammation in adipocytes and the effect of SDA on insulin signaling and insulin-stimulated glucose uptake in adipocytes. This study attempts to provide theoretical clues for the future application of SDA in the treatment of obesity and diabetes.

## Chapter 2 Review of Literature

### 2.1 Obesity

Obesity can be defined as a medical condition of excessive body fat, which may have an adverse effect on health. Body mass index (BMI) is an indirect measure of obesity. According to the World Health Organization (WHO), a BMI  $\geq 25$  kg/m<sup>2</sup> is defined as overweight, and a BMI  $\geq 30$  kg/m<sup>2</sup> is defined as obesity (35). With the increasing prevalence in adults and children, obesity is regarded as one of the most serious public health problems of the 21st century (36). In the United States alone, it is estimated that approximately 66% of all adults are overweight and approximately 32% are obese (36). In Europe, more than 50% of the adult population is overweight and 20-30% is obese. In addition, the prevalence has doubled or even risen threefold in less than two decades (37).

Aside from being a social stigma, obesity is recognized as a risk factor in the development of various clinical disorders, such as hypertension, osteoarthritis, dyslipidemia, cardiovascular disease, and certain types of cancer (4). Furthermore, obesity is frequently associated with insulin resistance, which in turn is linked to the development of type 2 diabetes (38). The risk of developing type 2 diabetes increases 27-fold for women with a BMI of 30 kg/m<sup>2</sup> and 93-fold for those with BMI  $\geq 35$  kg/m<sup>2</sup> relative to women with a BMI  $< 22$  kg/m<sup>2</sup> (39). Similarly, Chan et al. (40) found that men with a BMI  $\geq 35$  kg/m<sup>2</sup> had a 42-fold higher risk of diabetes compared with men with a BMI  $< 23$  kg/m<sup>2</sup>. In the SEARCH for Diabetes in Youth study (41), the prevalence



of overweight was 10.4% and obesity was 79.4% among 3959 youth with type 2 diabetes, illustrating that obesity is a major contributing factor in type 2 diabetes.

Although a few cases are caused solely by genes, endocrine disorders, medications, or psychiatric illness (42), obesity is most commonly caused by a combination of excessive energy consumption through dietary intake and inadequate energy expenditure. The primary treatment for obesity is dieting and physical exercise. Pharmaceutical interventions that reduce appetite or inhibit fat absorption and surgical options have also been adopted to address obesity.

## **2.2 Diabetes**

Diabetes is a complex metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes is divided into two main types, type 1 and type 2. Type 1 diabetes results from beta cell destruction, usually leading to absolute insulin deficiency. Type 2 diabetes, which accounts for at least 90% of all cases of diabetes, is characterized by defective insulin action, ranging from predominant insulin resistance with relative insulin deficiency, to a predominantly insulin secretory defect with insulin resistance.

Diabetes has become an epidemic and remains a major public health issue. Globally as of 2010 it is estimated that there are 285 million people with diabetes. In the United States, it is currently estimated that 25.8 million children and adults or 8.3% of the population have diabetes. In 2007, the total cost of diabetes was estimated to be \$174 billion (43). Type 2 diabetes is a common and serious condition that is associated with morbidity and reduced life expectancy. Type 2 diabetes is the leading causes of blindness, renal failure and lower limb amputation, and is an independent risk factor for cardiovascular disease (CVD) and is now considered to be one

of the leading causes of death (44). The prevalence of type 2 diabetes is associated with the adoption of a Western-type lifestyle, which has resulted in populations going to a diet high in saturated fat and sugar, as well as a reduction in physical activity.

## **2.3 N-3 polyunsaturated fatty acids**

### **2.3.1 The beneficial effects of n-3 PUFA on diabetes**

A healthful diet that avoid excess energy intake is a key component in the prevention and management of type 2 diabetes. A wealth of evidence indicates that consumption of cold-water marine fish or dietary fish oils containing n-3 long chain polyunsaturated fatty acids (LCPUFA) is associated with positive effects on obesity, inflammation, insulin resistance, and type 2 diabetes (45-47). The n-3 PUFA in fish oil refers specifically to eicosapentaenic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) and usually termed LCPUFA.

Diets high in saturated fatty acids have generally been associated with increased adiposity, reduced insulin sensitivity and greater risk of type 2 diabetes (48, 49). Switching a diet high in saturated fatty acids to one high in PUFA changed abdominal fat distribution and improved insulin sensitivity in 17 subjects including 6 people with type 2 diabetes, 6 non-obese and 5 obese people without diabetes (50). In a study of 84 obese patients, the degree of obesity and central distribution of body fat were inversely associated with the monounsaturated and n-3 long chain PUFA in adipose tissue (51). These observations indicate that a higher n-3 PUFAs status is associated with reduced abdominal obesity, the type of obesity highly correlated with insulin resistance and type 2 diabetes.

A considerable number of studies have demonstrated the beneficial effects of n-3 PUFAs on lipid related disorders in humans. Sirtori and colleagues (52) examined the effects of n-3 fatty acids on 953 patients with hypertriglyceridemia and different degrees of glucose intolerance. One-year treatment of 2 to 3 g/day n-3 ethyl esters was associated with a significant progressive reduction of triglyceridemia and an increase on high-density lipoprotein (HDL) levels, without apparent harmful effects on glycemic control. Similarly, another clinical trial with 30 patients with type 2 diabetes and hypertriglyceridemia showed that supplementation of EPA (465mg) and DHA (375mg) in capsules for 12 weeks significantly decreased triacylglycerol (TG) levels and non-HDL cholesterol levels with an increase in HDL cholesterol levels (53). In the study conducted by Kesavulu et al. (2002) (54), 34 non-insulin dependent diabetic patients were initially treated with antidiabetic drugs alone for one month, followed by supplementation with 1.8 g/day n-3 fatty acids along with antidiabetic drugs for two months. The combined treatment significantly reduced serum triglycerides from  $2.07 \pm 0.94$  mmol/l before combined therapy to  $1.54 \pm 0.49$  mmol/l after combined therapy and significantly increased HDL-cholesterol levels from  $0.93 \pm 0.099$  mmol/l before combined therapy to  $1.04 \pm 0.098$  mmol/l after therapy. In summary, n-3 fatty acids have been shown to improve lipid profile by decreasing triglyceride levels as well as increase HDL levels in subjects with type 2 diabetes or hypertriglyceridemia.

A diet rich in saturated and n-6 fatty acids leads to insulin resistance in rats. Replacement of 6 percent of the n-6 fatty acid linoleic acid in the diet with n-3 LCPUFA from fish oil prevented the development of insulin resistance, especially in the liver and skeletal muscle (33, 34). Likewise, in male Wistar rats, a high-fat diet enriched in n-3 LCPUFA prevented the defect of insulin signaling in muscle (55). Epidemiologic studies have reported an inverse association between fish intake and impaired glucose tolerance and type 2 diabetes. In the Dutch and Finnish

cohorts of the Seven Countries Study, an increase in the consumption of fish was inversely related to 2-h glucose level during the 20-year follow-up (49). Another study also reported that fish intake was associated with reduced risk of developing impaired glucose tolerance in elderly people who were normoglycemic and free of clinical diabetes (56). However, in most intervention studies, enrichment of fish oil failed to affect insulin sensitivity in patients with type 2 diabetes (57-59).

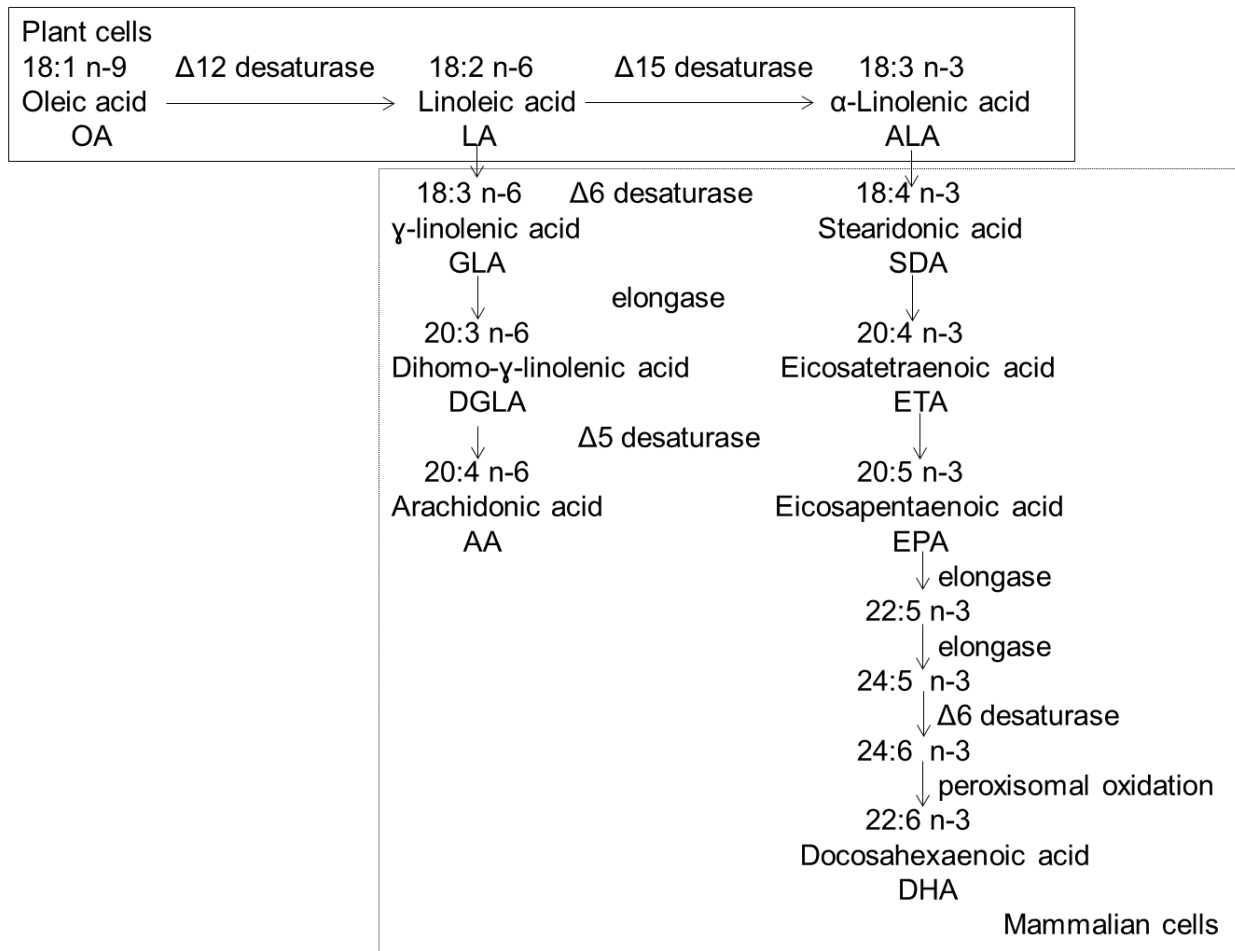
It is now accepted that inflammation is strongly related to obesity and the development of insulin resistance. N-3 PUFAs are known to have anti-inflammatory effects (60). In male C57BL/6J mice, EPA prevented and reversed insulin resistance in high-fat diet induced obese mice, and this effect appears to be mediated via modulation of adipose tissue inflammation, as indicated by decreased secretion of proinflammatory adipokines and increased secretion of adiponectin (54). Epidemiological evidence suggested that the intakes of n-3 PUFA were inversely associated with the levels of inflammation in healthy human subjects (61). In a clinical trial, n-3 PUFAs supplementation has been reported to decrease inflammation markers such as circulating C-reactive protein (CRP) and tumor necrosis factor (TNF)-alpha levels in patients with type 2 diabetes and hypertriglyceridemia (53). Likewise, in a study with overweight insulin-resistant women, 24 weeks of supplementation with n-3 PUFA improved circulating concentrations of interleukin-6 (IL-6) and CRP, and increased the levels of anti-inflammatory marker adiponectin (62). Taken together, supplementation with n-3 PUFA has anti-inflammatory effects in patients with type 2 diabetes and hypertriglyceridemia.

### **2.3.2 Dietary sources of n-3 PUFA**

Because n-3 and n-6 PUFA are essential fatty acids that cannot be synthesized de novo in mammals, they must be derived from the diets. A typical Western diet is high in n-6 and low in n-3 PUFA and some studies have suggested that increasing intake of n-3 fatty acids to adjust the n-6/n-3 ratio to 4:1 may enhance overall human health (63). The primary source of n-3 PUFA EPA and DHA is fish and fish oil, especially oily fish such as sardines, salmon, mackerel and tuna (64). The American Heart Association (AHA) has recommended that all adults intake 400-500mg /day of EPA and DHA to obtain the health benefits. This may be reached by consuming at least two serving of oily fish per week (65). Further, the AHA recommends 2-4 g/day of DHA and EPA for patients with hypertriglyceridemia (66). To achieve this recommendation, a fourfold increase in fish consumption would be required (66). However, there has been stagnation or decline in global fisheries (9). Aquaculture output has steadily grown and provided opportunities to meet increasing demands on wild fish population. However, fish meal and fish oil containing n-3 fatty acids is required in the diet of farmed fish to confer the documented health benefit to the consumers, which causes similar supply pressures on wild fish population. In addition to issues of sustainability, there is an increasing alarm over levels of methyl mercury in some species of long-lived fish (67). Furthermore, many individuals cannot tolerate the taste or smell of fish oils, even when provided in capsules. Oil from fermentation of microalgae can be very high in EPA or DHA. However, it is not currently being produced in sufficient quantities for wide-scale impact (1).

Another possibility is to increase the consumption of land-based sources of n-3 PUFA. Currently,  $\alpha$ -linolenic acid (ALA; 18:3 n-3) is the main n-3 fatty acid available in vegetable oils. A high concentration of ALA is found in flax seed oil, and lower concentrations are found in canola and soybean oil. ALA can go through elongation and desaturation to the longer chain n-3

fatty acid EPA (Fig 1). However, since the initial enzyme  $\Delta 6$  desaturase in the conversion of ALA to EPA is rate limiting in humans, there is poor conversion (0.1-7%) of ingested ALA to EPA as shown in some studies (14, 68). The degree of conversion of ALA to EPA also depends on the amount of linoleic acid (LA; 18:2 n-6) in the diet, since LA competes with ALA for  $\Delta 6$ -desaturation and diminishes the conversion of ALA to EPA. Due to the low rate of conversion of ALA to EPA, there is insufficient evidence that ALA promotes health through the conversion to EPA. Hence, there is a need and desire to identify and develop alternative sources of n-3 LCPUFA.



**Fig.1.** Metabolic pathway of n-6 and n-3 PUFA. (Adapted from Urisn 2003 (1) and Whelan 2009 (2) )

## **2.4 Stearidonic acid**

The n-3 fatty acid stearidonic acid (SDA; 18:4 n-3) is a metabolic intermediate between ALA and EPA, and it could effectively bridge the gap between the sustainability of a land-based oil and the effectiveness of fish oil. SDA is a PUFA with an 18-carbon chain and four double bonds in the acyl chain. Its molecular weight is 276.4, and its melting point is -57 °C (69).

### **2.4.1 SDA metabolism**

SDA represents the  $\Delta 6$  desaturation product of ALA, and thus bypasses a rate-limiting step in the conversion of dietary ALA to EPA. When SDA enters the metabolic pathway after the  $\Delta 6$  desaturase step, it is rapidly converted to EPA. The relative effectiveness with which dietary SDA is bioconverted to EPA is thus dependent upon regulation of the subsequent desaturation and elongation reactions (Fig. 1). Some studies have shown that consumption of SDA increased EPA levels in plasma, neutrophil, heart, liver, and erythrocyte phospholipids (12-15). When dogs were supplemented with SDA and EPA ethyl ester for 12 weeks, SDA was 20-23% as efficient in raising erythrocytes and heart EPA levels as compared with dietary EPA (13). Similar results were shown in a clinical study conducted by the same group, dietary SDA increased red blood cell EPA content by about 17% (14). James et al. (12) assessed the relative efficiencies of dietary SDA, ALA, and EPA in elevating plasma EPA. In this study, dietary SDA was found to increase plasma EPA between threefold and fourfold more efficiently than comparable levels of ALA and was approximately one-third as effective as dietary EPA. By the relative effectiveness of conversion of dietary SDA to EPA, SDA consumption would be expected to confer the health benefits associated with consumption of EPA. SDA has been targeted as a potential biologically active surrogate for EPA, due to its relatively efficient conversion following consumption.



### 2.4.2 SDA function

Experimental and clinical studies indicate that SDA shares many of the biological effects of dietary EPA. Recently, several studies established the emerging roles of SDA on inflammation, hypertriglyceridemia, cardiovascular diseases, and cancer. These findings suggest that SDA could become a prominent surrogate for EPA in health promotion and disease prevention.

In a study by Surett et al. (11), mildly hypertriglyceridemic humans were instructed to consume SDA (1.88 g/d) in the form of Echium oil. After 4 weeks, serum triacylglycerol concentration decreased by 21% compare with baseline (11). In contrast, in overweight healthy subjects, plasma triacylglycerols or other lipids were not affected by SDA-soybean oil (3.7 g/d) or EPA ethyl esters (1 g/d) treatment for 16 weeks (14). In mildly hypertriglyceridemic mice (apoB100-only LDLr KO), a diet containing 10% of calories as echium oil increased plasma and liver lipid EPA level and decreased plasma TG, very-low-density lipoprotein (VLDL) lipid concentration, and hepatic TG content compared to the palm oil diet. Similar results were obtained in mice fed fish oil diets. In addition, down regulation of several genes involved in hepatic TG bio-synthesis was similar for mice fed echium oil and fish oil, which suggests that the reduction in plasma triacylglycerol concentrations was through decreased transcription of genes important in hepatic lipid synthesis (70). Further studies from this lab indicated that echium oil diets reduced atherosclerosis in B100-LDLrko mice, and this results were comparable to that of fish oil diets (71).

Omega-3 index is a new marker for cardiovascular disease. This is the sum of EPA and DHA in erythrocyte membranes expressed as a percentage of total erythrocyte fatty acid. The

omega-3 index has been proposed as a risk factor for a variety of cardiovascular disease endpoints (13, 72). A clinical study has found that SDA and EPA ethyl esters are more effective than ALA on changing the omega-3 index (14). Relative to EPA, SDA increased red blood cell EPA by approximately 17%, whereas the efficiency of ALA was approximately 0.1% (14). Since higher omega-3 indexes have been associated with a reduction in risk for sudden cardiac death, the impact of SDA intake on the omega-3 index may suggest its beneficial effect on cardiovascular diseases.

When Balb/c mice were fed diets with purified triacylglycerol containing 10% (w/w) of ALA, SDA, or EPA, the release of the inflammatory mediators TNF $\alpha$  and prostaglandin E2 (PGE2) were measured in whole blood and splenocytes stimulated with lipopolysaccharide (LPS). All three n-3 PUFA inhibited TNF $\alpha$  production in whole blood and PGE2 production in splenocytes. There was no difference among the dietary n-3 PUFA (17). In a mouse ear inflammation model (73), SDA and EPA isolated from the seaweed *Undariapinnatifida* showed inhibitory effects against the inflammatory symptoms of edema, erythema, and blood flow. In healthy humans, low-dose supplementation of dietary n-3 PUFA including 49mg/d of SDA in capsule influenced the expression of cytokine and proinflammatory lipid mediators in whole blood after lipopolysaccharide stimulation ex vivo (74). On the contrary, the study of James et al. (2003) (12) showed that consumption of 0.75g and then 1.5g SDA or EPA as ethyl esters per day for period of 3 weeks each had no effect on production of TNF $\alpha$  and IL-1  $\beta$  by LPS-stimulated whole blood. In another study, supplementation of SDA (0.13 g/d) to healthy subjects in the form of blackcurrant seed oil reduced prostaglandin E2 production and caused a moderate immune-enhancing effect, but no effects were observed on the production of proinflammatory cytokines IL-1 $\beta$  and IL-2 (75).

In APC<sup>min/+</sup> mice, a genetic model of colorectal cancer, dietary SDA ethyl esters at a level of 3 g/100g diets reduced tumor multiplicity by ~50%, and this effect was as effective as EPA, whereas DHA was less effects were observed in mice fed DHA ethyl esters (18). In this study, dietary ALA as ethyl esters has no effect on tumor number and size (18). In NIH-3T3 cells, cell proliferation was slightly stimulated in the presence of ALA but was strongly inhibited in the presence of SDA at the same concentration over a 4-d period (76). This study concluded that this inhibition may be caused by enhanced lipid peroxidation as a result of the high levels of SDA present. Cyclooxygenase-2 (COX-2), the inducible enzyme that catalyzes prostaglandin production has been used as a biomarker for human breast cancer. A study showed that in the MDA-MB-231 breast cancer cell line, SDA treatment as ethyl esters significantly decreased the level of COX-2 protein, but ALA had no effect on COX-2 protein expression (77). In another study, in a xenograft model for the recurrence of prostate cancer, decreased proliferation index and increased apoptotic index were observed in recurring prostate tumors in mice fed with high n-3 fat diet containing 15 wt.% SDA oil (60% SDA), and this diet also caused a dramatic decrease in the growth of tumors (78). This study also provided evidence that SDA inhibition of tumorigenesis may be due to competition with n-6 fatty acids LA and AA for 15-lipoxygenase-1 (LO-15-1) and COX-2 activities to produce prostaglandin, which is positively correlated with tumor number. All of these studies indicate SDA is more effective than its metabolic precursor ALA as a protecting agent in tumorigenesis.

### **2.4.3 Dietary source of SDA**

SDA is a minor component of several fish oils, contributing 0.5-4% of the total fatty acids (1, 79). Hence, fish oils are used mainly as sources of EPA and DHA instead of SDA. SDA is also found in the seed oil and leaves of a number of plants, including blackcurrant

(*Ribesnigrum*), borage (*Boragoofficinalis*), evening primrose (*Oenotherabiennis*), and *Echiumplantagineum*. Echium oil from the seeds of *Echium plantagineum* has been identified as a natural source enriched in SDA, which account for approximately 12% of total fatty acids in the oil (11). However, these plants are not adapted to wide scale cultivation, and oil yields are low and variable. One approach to achieving SDA production on a large scale is to transfer the biosynthetic pathway of SDA directly into a high oil-yielding oilseed crop where existing commercial cropping and oil extraction technologies are utilized.

The use of transgenic technology in oil seed crops offers the opportunity to modify and tailor the composition of seed oils. Recently, genes for  $\Delta 6$  and  $\Delta 15$  desaturases enzymes have been introduced into canola and soybeans to produce SDA in vegetable oils. Through a single desaturation step (Fig. 1), the *Boragoofficinalis* L.  $\Delta 6$  desaturase can convert LA and ALA to  $\gamma$ -linolenic acid (GLA; 18:3 n-6) and SDA, respectively. In the soybean transfected with cDNA of  $\Delta 6$  desaturase, the average GLA levels ranged from 3.4 up to 28.7%, while the SDA levels varied from 0.6 to 4.2% (80). In conventional canola oil, the primary fatty acid is oleic acid (OA; 18:1 n-9). Thus, the production of significant quantities of SDA in canola oil requires increased flux through the PUFA pathway from OA to ALA and then the addition of  $\Delta 6$  desaturase activity (Fig. 1). By generating transgenic canola (*Brassica napus*) lines that are expressed in seeds, the  $\Delta 6$  and  $\Delta 12$  fatty acid desaturases isolated from the commercially grown fungus *Mortierellaalpina* and the  $\Delta 15$  fatty acid desaturase from canola. Seed oil from independent transformants accumulated SDA up to 23% of the oil by weight (1). Dietary intake of n-3 fatty acids would increase significantly if SDA was produced in soybean and canola oil since it is not present in these vegetable oils.

This genetic SDA soybean oil has been used in a recent study. Hammond et al. (2008) (81) performed a 28-day gavage study and a 90-day/one generation reproduction feeding study on rats to assess the safety of genetic soybean oil. There was no evidence of treatment-related adverse effects up to the highest dosages of SDA soybean oil tested. This study confirmed the safety of SDA and the other fatty acid components present in SDA soybean oil administered.

## **2.5 Obesity and adipocyte differentiation**

Adipose tissue is mainly composed of adipocytes that store energy in the form of triacylglycerol during energy excess and break down this stored lipid into free fatty acids during energy deprivation (82). Obesity arises from the imbalance between energy intake and energy expenditure, leading to a pathological accumulation of adipose tissue. This development of adipose tissue is caused by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes. The size of adipocytes increases because of increased storage of triacylglycerols from dietary sources or endogenous lipogenic pathways. The number of adipocyte increases as a result of increased proliferation and differentiation (19). Abnormal differentiation of preadipocytes affects glucolipid metabolism, induces obesity and insulin resistance, and results in type 2 diabetes. Consequently, understanding the development and regulation of adipogenesis should provide valuable information for the prevention and treatment of obesity and type 2 diabetes.

### **2.5.1 Cell models for studying adipocyte development**

Cell culture models have been extensively used to study adipogenesis. Two types of cell lines have served as useful models to study this process. These cell lines are fibroblastic multipotent cell lines that have not undergone commitment to the adipocyte lineage and

preadipocyte cell lines that have undergone commitment. When appropriately induced, these cell lines differentiate into adipocyte (83). 3T3-L1 and 3T3-F442A are the two most frequently employed preadipocyte cell lines for studying adipocyte differentiation. These cell lines were derived from disaggregated mouse embryo cells and were selected for their ability to accumulate triacylglycerol lipid accumulation (84, 85). Although committed to the adipocyte lineage, proliferating 3T3-L1 preadipocytes exhibit characteristics similar to those of other 3T3 fibroblasts. Upon exposure to the adipogenic inducers, 3T3-L1 preadipocytes can be induced to differentiate into adipocytes that display the morphological and biochemical characteristics of adipocytes in situ (86). Agents which are efficient inducers of adipocyte differentiation include fetal bovine serum (FBS), dexamethasone, isobutylmethylxanthine, and insulin. These agents are most effective when used in combination.

The adipogenic program in preadipocyte cell lines consists of several sequential events that have been well characterized. The first step of adipocyte differentiation in cell culture models involves growth arrest at confluence. At this stage, cells arrest in the  $G_0/G_1$  stage of the cell cycle (86). When appropriate adipogenic stimuli are presented, growth-arrested cells will synchronously re-enter the cell cycle and undergo several rounds of cell division, referred to as mitotic clonal expansion (20). It has been suggested that DNA replication and the accompanying remodeling of chromatin structure increase the accessibility of cis-elements to trans-acting factors, which activate or derepress transcription of the gene(s) that gives rise to the adipocyte phenotype. During these mitotic events, preadipocytes express high levels of CCAAT/enhancer-binding protein (C/EBP)  $\beta$  and C/EBP $\alpha$  (87, 88). Numerous studies have shown that blocking clonal expansion with the drugs rapamycin, aphidicolin, or roscovitin disrupts differentiation (89-91). This study demonstrates that mitotic clonal expansion is a

prerequisite for terminal differentiation. As the cells complete clonal expansion, they enter the terminal maturation process where they acquire all the specialized equipment of adipocytes. Two important transcription factors, peroxisome proliferator-activated receptor (PPAR)  $\gamma$  and C/EBP $\alpha$ , are involved in maintaining terminal differentiation (92). These transcription factors together stimulate the expression of adipocyte specific genes such as lipoprotein lipase (LPL), leptin, glucose transporter 4 (GLUT4), stearoyl-CoA desaturase 1 (SCD-1) and adipocyte fatty acid binding protein (aP2) (93), thereby leading to the characteristic phenotype of the mature adipocyte.

### **2.5.2 Transcriptional regulation of adipogenesis**

Several classes of adipogenic transcriptional factors such as PPAR $\gamma$ , C/EBPs, and Sterol regulatory element binding protein-1c (SREBP-1c) appear to be essential in the control of adipocyte differentiation. These factors are involved in the sequential expression of adipocyte-specific proteins such as GLUT4, LPL, SCD-1, and aP2, which mediate fatty acid or glucose uptake into adipocytes, triglyceride hydrolysis, and lipogenesis (21, 22). Thereafter, adipocytes incorporate glucose and free fatty acids to synthesize and accumulate lipids as energy, resulting in an increase in cell size.

PPAR $\gamma$  is a member of the nuclear hormone receptor superfamily. PPAR $\gamma$  is expressed as three isoforms, PPAR $\gamma$ 1, PPAR $\gamma$ 2, and PPAR $\gamma$ 3. PPAR $\gamma$ 1 is widely expressed, including expression in adipose tissue, whereas PPAR $\gamma$ 3 has been found in adipose tissue, large intestine, and macrophages. PPAR $\gamma$ 2 is expressed fairly specifically in adipose tissue, suggesting that the PPAR $\gamma$ 2 form is most important for adipocyte physiology (94). Similar to a subset of other nuclear receptors, PPAR $\gamma$  binds as heteromeric complexes with the retinoid X receptor (RXR)

to PPAR response elements (PPRE/DR-1; direct repeats of the sequence RGGTCA separated by one base) (95). PPAR $\gamma$  is a central regulator during adipogenesis. The transcription factor is induced during differentiation and is responsible for activating a number of genes involved in fatty acid binding, uptake and storage, including ap2, LPL, acyl coenzyme A synthase, and phosphoenol pyruvate carboxykinase (PEPCK) (83). Furthermore, PPAR $\gamma$  decreases the expression of the adipocyte-derived signaling molecule leptin, which results in an increase in energy intake and optimization of energy usage, contributing further to PPAR $\gamma$ 's adipogenic effect (87).

Nuclear hormone receptor superfamily members are ligand-activated transcription factors, and PPAR $\gamma$  is no exception. Fatty acids and their derivatives are regarded as natural ligands for PPAR $\gamma$ . The prostaglandin, 15-deoxydelta12,14-prostaglandin J2, is a potent activator of PPAR $\gamma$ . This eicosanoid is able to bind to and activate PPAR $\gamma$ , and can promote adipogenesis when added to cultured fibroblasts (96, 97). However, this prostaglandin was shown to be present at low levels in adipose tissue. Thiazolidinediones (TZDs) such as troglitazone, rosiglitazone and pioglitazone are high-affinity synthetic ligands for PPAR $\gamma$  and were used clinically as antidiabetic drugs (98). The ability of these drugs to activate PPAR $\gamma$  correlates well with their ability to induce adipocyte differentiation, as well as their hypoglycemic activities in rodents and humans. However, the endogenous ligand of PPAR $\gamma$  is currently unknown. SREBP-1c is implicated in stimulating endogenous PPAR $\gamma$  ligand production (99).

The C/EBPs belong to the basic-leucine zipper class of transcription factors. Three members of the C/EBP family of transcription factors-C/EBP $\alpha$ ,  $\beta$  and  $\delta$  are expressed at defined times during adipogenesis, and each plays a specific regulatory role in adipocyte differentiation. In cultured preadipocytic cell lines, C/EBP $\beta$  and  $\delta$  are expressed early and transiently in response



to an adipogenic cocktail (89). These isoforms then activate the expression of PPAR $\gamma$ 2 and C/EBP $\alpha$  in the preadipocyte, subsequently triggering full-blown adipocyte differentiation. Ectopic expression of C/EBP $\beta$  in 3T3-L1 preadipocytes is sufficient to induce differentiation in the absence of hormone. Overexpression of C/EBP $\delta$  can accelerate adipogenesis with the addition of hormonal inducers (100). Embryonic fibroblast cells derived from C/EBP $\beta$  and  $\delta$  knockout mice do not differentiate into mature adipocytes and do not express C/EBP $\alpha$  and PPAR $\gamma$  (101). In addition, mice lacking both  $\beta$  and  $\delta$  exhibit dramatic reduction of brown adipose tissue (BAT), due to decreased lipid accumulation and smaller decreases in white adipose tissue (101). These experiments illustrated the important roles of C/EBP  $\beta$  and  $\delta$  in adipocyte differentiation.

C/EBP $\alpha$  is expressed late in the differentiation program and is responsible for the expression of many adipocyte marker genes. Several adipocyte-specific genes, such as those encoding SCD-1, GLUT-4, aP2, PEPCCK and UCP, have C/EBP-binding sites in their promoters (22). The conditional expression of this isoform in 3T3-L1 fibroblasts stimulates adipogenesis in the absence of any hormonal induction. In addition, when production of the C/EBP $\alpha$  protein was inhibited by expression of antisense C/EBP $\alpha$  mRNA, lipid accumulation and expression of fat-specific marker genes were inhibited (102). Moreover, adipocytes from mice in which the C/EBP $\alpha$  gene was disrupted have defects in lipid accumulation (103). These observations establish the requirement for C/EBP $\alpha$  in preadipocyte differentiation.

SREPBs are basic helix-loop-helix-leucine zipper transcription factors. Adipocyte determination and differentiation-dependent factor 1(ADD1) was cloned from a rat adipocyte cDNA library and is homologous to human SREBP-1c (104). It was isolated independently as an adipocyte-associated transcription factor and as a sterol metabolism regulatory factor based on its

ability to bind to two distinct sequences: the E-box (ATCACGTA) and the sterol regulatory element (ATCACCCAC), respectively. ADD1/SREBP-1c is responsible for the transactivation of lipogenic genes that encode enzymes of triacylglycerol synthesis, as well as fatty acid synthesis, desaturation, and uptake. The expression of ADD1/SREBP-1 is induced within the first 24h after adipogenic hormones are added. This expression appears to be important for development of the adipocyte phenotype, as introduction of a dominant-negative mutant markedly inhibited differentiation of 3T3-L1 preadipocytes (105). Ectopic expression of ADD1/SREBP-1 is not sufficient to convert NIH-3T3 cells into adipocytes (105). However, a recent study suggested that the retroviral expression of ADD1 in 3T3-L1 preadipocytes increases the expression of PPAR $\gamma$  (106). Kim et al (99) showed that simultaneous ADD1 expression increases the transcriptional activity of PPAR $\gamma$  by increasing the formation of the endogenous ligand for PPAR $\gamma$ .

## **2.6 Insulin signaling transduction**

Insulin is an anabolic hormone that is synthesized and secreted by pancreatic beta cells. Insulin mediates a wide spectrum of biological responses. Insulin increases glucose uptake in muscle and fat and inhibits hepatic glucose production, thus serving as the primary regulator of blood glucose concentration. Insulin also stimulates glycogen, lipid and protein synthesis and inhibits lipolysis, glycogenolysis, and protein breakdown. Moreover, insulin acts on certain cell types as a growth factor to modulate cell growth and differentiation (107). These myriad effects are initiated by the binding of insulin to its receptor.

The insulin receptor (IR) is a tetrameric protein consisting of two  $\alpha$ - and two  $\beta$ -subunits that function as allosteric enzymes in which the  $\alpha$ -subunit inhibits the tyrosine kinase activity of

the  $\beta$ -unit. The  $\alpha$ -subunit is located entirely at the extracellular face of the plasma membrane and contains the insulin binding site, whereas the  $\beta$ -subunit is a transmembrane peptide. The insulin molecule binds to the  $\alpha$  subunit of the receptor, releasing the inhibition of tyrosine autophosphorylation by the  $\beta$  subunit (108). The activated IR then phosphorylates the insulin receptor substrates family (IRS-1 to -4) and other substrates including Gab-1, p60<sup>dok</sup>, Cbl, and APS (109). There are currently four members of the IRS family known to be involved in insulin signaling, with IRS-1/2 being the most important for glucose uptake (110). IRS proteins contain a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain at the N terminus, which appear to anchor the IRS proteins to the activated insulin receptor. The C terminal region of IRS-1 contains a number of consensus tyrosine phosphorylation motifs, which are phosphorylated directly by the IR. Tyrosine-phosphorylated IRS-proteins generate downstream signals by the direct binding to the SH2 domains of various signaling proteins, including phosphatidylinositol 3 (PI3)-kinase, SHP2, Shc, Gab2, Nck, and Crk (111). The three major pathways emanating from activated IRS include the IRS/PI3-kinase pathway, the Cbl-associated protein (CAP)/Cbl pathway, and the (RAS)/mitogen-activated protein kinase (MAPK) pathway (Fig. 2), with the two former pathways mainly involved in the positive control of insulin action.

### **2.6.1 PI3 kinase pathway**

PI3K is the main signal mediator of the metabolic and mitogenic actions of insulin. This enzyme consists of a p110 catalytic subunit and a p85 regulatory subunit with two SH2 domains that interact with tyrosine-phosphorylated pYMXM and PYXXM motifs in IRS proteins (112). After the association of p85 with IRS-1/2, the p110 subunit has increased catalytic activity. This allows phosphorylation of its substrate, phosphatidylinositol bisphosphate (PtdIns(4,5)P<sub>2</sub>), which

is then phosphorylated on the 3' position of the inositol ring yielding the second messenger Ptd(3,4,5)P3 (113). Ptd(3,4,5)P3 subsequently recruits phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (also known as AKT) to plasma membrane, and this co-localization allows PDK1 to phosphorylate AKT at Thr308. Full activation of AKT occurs upon phosphorylation of Ser473 by PDK2 or DNA-PK (DNA-activated protein kinase) (114).

Akt is one of the critical targets playing a pivotal role in mediating insulin-responsive metabolic actions. Activation of the PI3 kinase-AKT pathway leads to the stimulation of glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, from intracellular storage to the plasma membrane (115). Expression of a constitutively active AKT in 3T3-L1 adipocytes induced glucose uptake in the absence of insulin by stimulating the translocation of GLUT4 to the plasma membrane (116, 117). On the contrary, insulin-stimulated GLUT4 translocation is inhibited by expression of a dominant-interfering AKT mutant (118, 119). The activation of the PI3 kinase-AKT pathway also mediates other insulin-induced response. AKT is able to phosphorylate glycogen synthase kinase 3 beta (GSK3 beta), thereby decreasing the activity of this kinase. As a result, AKT (PKB) abolishes the inhibition of glycogen synthesis and activates translation via the regulatory activity of the initiation factor of translation eukaryotic translation initiation factor 2 (eIF2). Activated Akt also induces protein synthesis via mTOR signaling, and stimulates lipogenesis via up-regulation of the expression of the fatty acid synthase gene (120). In addition, the activation of AKT has been reported to regulate cell survival and growth through inhibition of several pro-apoptotic agents (121).

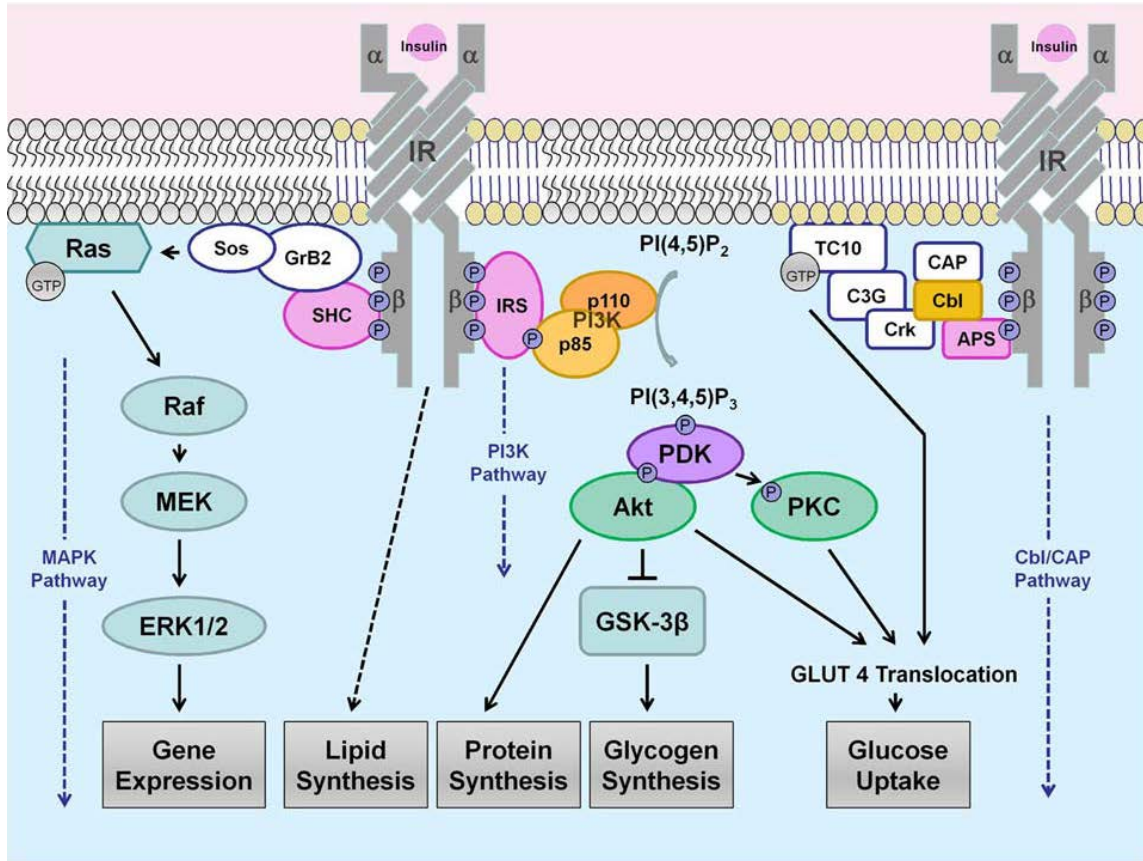
### **2.6.2 Cbl/CAP pathway**

In addition to PI(3)K activity, there is another signaling pathway that seems to be required for insulin-stimulated glucose uptake. This pathway involves tyrosine phosphorylation of the protooncogene Cbl. Cbl is recruited to the insulin receptor by interaction with the adapter protein C-Cbl-associated protein (CAP), which contains three adjacent SH3 domains and a sorbin homology (SoHo) domain (122). After tyrosine phosphorylation of Cbl, the CAP-Cbl complex translocates to the plasma membrane domain enriched in lipid rafts, mediated by the interacting of the SoHo domain of CAP with the protein flotillin (123). Translocation of phosphorylated Cbl recruits the adapter protein CrkII to the lipid raft via interaction of the SH2 domain of CrkII with phospho-cbl (124). CrkII also forms a constitutive complex with the guanine exchange factor C3G which specifically activates the small GTP binding protein Tc10 and ultimately the TC10 downstream effector TCGAP (125). TC10 is thought to act as a second signal for the GLUT4 protein that functions in parallel with the activation of the PI3K pathway.

### **2.6.3 The MAPK cascade**

Insulin signaling can also activate the mitogen-activated protein kinase (MAPK) signaling cascade, which leads to expression of various cellular proliferation and/or differentiation genes (110). The autophosphorylation on tyrosine residues of the insulin receptor promotes the formation of a Shc-Grb2 complex. This complex is recruited to the plasma membrane, where it associates with son-of-sevenless (SOS), a guanine nucleotide exchange protein that converts inactive GDP-Ras to active GTP-Ras. Once activated, Ras lead to a serine kinase cascade through the stepwise activation of Raf, MEK, and ERK. Activated ERK translocates into the nucleus, where it catalyzes the phosphorylation of many transcription factors that leads to cellular proliferation and differentiation (126). Although the MAPK pathway

is a well-characterized insulin signaling cascade, it is not involved in many of the important metabolic responses of the hormone (127).



**Fig. 2. Insulin signalling pathways.** The binding of insulin to the  $\alpha$ -subunit of insulin receptor (IR) results in an autophosphorylation and activation of  $\beta$ -subunit of the IR. The activated receptor phosphorylated cellular proteins such as members of the IRS family, Shc, CAP and Cbl. Upon tyrosine phosphorylation, these proteins interact with signalling molecules through their SH2 domains, resulting in activation of three main signalling pathways: the IRS/phosphatidylinositol 3 (PI3) kinase pathway; the (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway. These pathways act coordinately to regulate diverse biological effects of insulin including protein synthesis, lipid synthesis, glucose uptake, glycogen synthesis and gene expression. (Yang 2009 (128))

## **2.7 Obesity, insulin resistance and systemic inflammation**

Obesity and type 2 diabetes are strongly associated with insulin resistance. Insulin resistance is a pathophysiological state exhibiting combined inability of muscle and adipose tissue to facilitate glucose uptake and of the liver to suppress glucose output in response to increasing amounts of insulin (129). It is becoming clear that obesity promotes a chronic inflammatory state and eventually develops insulin resistance and related metabolic abnormalities (130). Various studies have shown that insulin resistance is tightly correlated to the impairment of insulin signaling resulting from negative modulation of downstream effector activities (131). The high incidence of obesity and type 2 diabetes and the seriousness of their clinic consequences make it imperative to understand the molecular basis of insulin signaling and insulin resistance.

### **2.7.1 The initiation of inflammation in obesity**

There is growing evidence that obesity, insulin resistance, and type 2 diabetes are tightly associated with abnormal inflammatory responses. These inflammatory responses include elevated secretion of pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), C-reactive protein, and interleukin-6 (IL-6), and increased activation of inflammatory signaling pathways such as the nuclear factor  $\kappa$ B (NF- $\kappa$ B) and Jun N-terminal kinase (JNK) systems in insulin targeted tissues of obese animals (132, 133). The inflammatory state associated with obesity appears to be triggered by and to reside predominantly in adipose tissue.

Adipose tissue plays a central role in maintaining lipid homeostasis and energy balance by storing triacylglycerol or releasing free fatty acid (FFA) in response to changes in energy demands. As individuals become obese and their adipocytes enlarge, adipose tissue undergoes



molecular and cellular alterations affecting systemic metabolism. Fasting whole-body FFA and glycerol release from adipocytes is increased in obese women compared with lean women (32). Physiological elevations of plasma FFA inhibit acutely as well as chronically insulin stimulated glucose uptake in a dose dependent fashion (32). FFA can activate at least three inflammation-associated proteins that are involved in the cross-talk between inflammatory and metabolic pathways. The FFA-induced activation of protein kinase C (PKC) delta, inhibitor kappaB kinase (IKK), or JNK leads to serine phosphorylation of the insulin receptor substrate (IRS)-1 which attenuates its ability to transduce insulin mediated cellular events (26, 27, 134).

Adipose tissue also plays an important role as an endocrine organ secreting different hormones and cytokines that can augment or impair whole-body insulin sensitivity. Adipose tissue in obese individuals shows increased expression of pro-inflammatory proteins including TNF- $\alpha$ , IL-6, MCP-1, and iNOS (25-27). Adiponectin is an adipose-specific plasma protein, which has an anti-inflammatory action (28). Plasma adiponectin concentrations are decreased in obesity and are positively associated with whole-body insulin sensitivity (135). Locally secreted chemokines like MCP-1 attract pro-inflammatory macrophages into the adipose tissue where they function to scavenge moribund adipocytes. These tissue macrophages are the main source of pro-inflammatory cytokines that further activate the inflammatory program in neighboring adipocytes, exacerbating inflammation and insulin resistance (136).

### **2.7.2 Inflammatory mediators**

Inflammatory cytokines TNF- $\alpha$ , and IL-6 are considered the main regulators in the development of insulin resistance. Both can activate the inflammation pathways JNK and IKK $\beta$ , then impair insulin action by interfering with insulin signaling (137). The first identified

molecular link between inflammation and obesity was TNF- $\alpha$ . The mRNA levels of TNF- $\alpha$  in adipose tissue of ob/ob mice are significantly increased compared with wild-type controls (138). Similarly, in humans, the expression of TNF- $\alpha$  in adipose tissue has also been shown to be increased in obesity and improvement of this increased TNF- $\alpha$  expression following weight loss (139). Mice lacking TNF- $\alpha$  or TNF receptors had improved insulin sensitivity in both dietary and genetic models of obesity (140). These studies indicate that TNF- $\alpha$  is an important mediator of insulin resistance in obesity.

IL-6 was among the first inflammatory cytokines to be implicated as a predictor or pathogenic mediator of insulin resistance and cardiovascular disease. Increased circulating IL-6 levels were observed in patients with obesity and type 2 diabetes (141, 142). Concentrations of IL-6 decreased in parallel with weight loss and improvement of insulin resistance in patients undergoing bariatric surgery (143). IL-6 is expressed in and released from both the subcutaneous and visceral adipose tissue. Visceral fat is more strongly linked to insulin resistance than subcutaneous fat depots (144) and has been found to secrete as much as 2- to 3-fold more IL-6 than subcutaneous fat (145). The liver is a likely target of IL-6 produced by adipose tissue. Several studies have suggested that IL-6 can inhibit hepatic insulin signaling in vitro and in vivo. Klover et al. (2003) (146) demonstrated that HepG2 cells and primary mouse hepatocytes have impaired insulin receptor signaling and insulin-dependent glycogen synthesis when acutely pretreated with IL-6. In mice, chronic exposure to IL-6 inhibits autophosphorylation of the insulin receptor and downstream signaling mediators in liver (147). On the contrary, depletion of IL-6 selectively improved hepatic insulin sensitivity in diet-induced obese mice (147, 148).

Adiponectin is mainly synthesized by adipocytes, and is considered a major anti-diabetic and anti-atherogenic adipokine (149-152). Plasma adiponectin concentrations are decreased in

obesity and are positively associated with whole-body insulin sensitivity (153). In accordance with these findings, the increased expression of adiponectin improves insulin sensitivity and weight loss (154, 155). Adiponectin exerts its insulin-sensitizing effect by inhibiting gluconeogenesis and stimulating fatty acid oxidation via activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)- $\alpha$  (156). In addition, adiponectin has been reported to stimulate production of nitric oxide, reduce expression of adhesion molecules in endothelial cells, and decrease cytokine production from macrophage by inhibition NF- $\kappa$ B signaling (135). Two receptors for adiponectin have been identified, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), and their expression levels are reduced in obesity, apparently in correlation with reduced adiponectin sensitivity (157). Yamauchi et al. (2007) (156) demonstrated that overexpression of either AdipoR1 or AdipoR2 in the liver ameliorated obesity-linked insulin resistance and diabetes in the db/db mice. In contrast, simultaneous disruption of AdipoR1 and R2 abolished adiponectin binding and actions, resulting in increased triglyceride content, inflammation, and oxidative stress, thus leading to insulin resistance and marked glucose intolerance.

Monocyte chemoattractant protein-1 (MCP-1, also known as chemokine ligand 2, CCL2) is one of the key chemokines responsible for monocyte recruitment into adipose tissue. It activates the resident macrophages to secrete cytokines and chemokines to recruit additional monocytes into fat, resulting in a vicious cycle amplifying the inflammatory status. MCP-1 gene expression in adipose tissue is increased in genetically obese diabetic (db/db) mice and high-fat diet-induced obesity mice (136). Mice engineered to express an MCP-1 transgene in adipose tissue exhibited insulin resistance, macrophage infiltration into adipose tissue, and increased hepatic triglyceride content. Conversely, disruption of the MCP-1 gene reduced the extent of

macrophage accumulation in adipose tissue, insulin resistance, and hepatic steatosis associated with obesity (136). Consistent with this, mice deficient in CCR2, a major cell surface receptor for MCP-1, have reduced macrophage content of adipose tissue and improved systemic glucose homeostasis and insulin sensitivity (158). These studies convincingly showed that the MCP-1 contributes to macrophage recruitment to adipose tissue and systemic insulin resistance.

### **2.7.3 Inflammatory pathways underlying insulin resistance**

How do inflammatory cytokines and fatty acids mediate insulin resistance? The inhibition of downstream components of the insulin receptor signaling pathways is relevant to insulin-mediated glucose metabolism and cross-talk with inflammatory signaling. Phosphorylation of tyrosine residues of IRS-1 upon activation of the insulin receptor constitutes a critical step in the transmission of the insulin signaling to downstream effectors and biological outcomes. It is well established that insulin-stimulated tyrosine phosphorylation of IRS-1 is reduced in animal models of insulin resistance and type 2 diabetic patients. Exposure of cells to inflammatory cytokines such as TNF $\alpha$  or elevated free fatty acids stimulates phosphorylation of IRS-1 on its serine (ser) residues (159-161). Serine phosphorylation of IRS-1 reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor and thereby inhibits downstream signaling and insulin action (159, 161, 162). Several serine/threonine kinases are activated by inflammatory or stressful stimuli and contribute to inhibition of insulin signaling, including JNK, IKK, and PKC.

JNK1 is a key mediator of environmental stresses and inflammation. JNK1 expression is activated by FFAs and TNF- $\alpha$  (163). Upon activation, JNK1 associates and phosphorylates IRS-1 on Ser307 (corresponding to Ser312 in humans) that is located at the end of the PTB domain of

IRS1 (161), a domain involved in the binding of IRS1 to the IR. The phosphorylation of Ser307 uncouples IRS1 from the IR, decreases the tyrosine phosphorylation of IRS-1, and impairs further insulin signaling (164). In obesity, JNK activity was increased in the liver, muscle, and fat tissues. Conversely, an absence of JNK1 resulted in decreased adiposity, increased insulin sensitivity, and enhanced insulin receptor signaling capacity in both genetic and dietary-induced mouse models of obesity (27). Liver-specific knockdown of JNK1 in obese diabetic mice lowered circulating glucose and improved insulin sensitivity, leading to the development of IR (165).

IKK $\beta$  is another key inflammatory pathway involved in insulin action. It has been reported to affect insulin signaling through at least 2 pathways. IKK $\beta$  has been observed to interact with IRS-1 through phosphorylation at the serine residue (166). Alternatively, IKK $\beta$  can phosphorylate the inhibitor of NF- $\kappa$ B (nuclear factor- $\kappa$ B), thus activating NF- $\kappa$ B, a transcription factor that stimulates the production of inflammatory molecules such as TNF- $\alpha$  and interleukin-6, which further activate JNK and NF- $\kappa$ B pathways through a feed-forward mechanism (134, 167). IKK $\beta$  heterozygous knockout mice (IKKb $^{+/-}$ ) are partially protected against insulin resistance due to a high-fat diet or genetic obesity (168, 169). Pharmacologic inhibition with salicylates, presumably through the targeting of IKK $\beta$ /NF- $\kappa$ B, has proved to increase insulin resistance in obese mice models (169). Yuan et al. (2001) (168) showed that TNF $\alpha$  treatment in 3T3-L1 adipocytes induces insulin resistance and that this could be prevented by pretreatment with the IKK $\beta$  inhibitor, aspirin.

PKC- $\theta$  plays essential roles in the fatty-acid induced insulin resistance. Evidence suggests that circulating FFA concentration was elevated in obese subjects compared with their lean counterparts (47, 170). Lipid infusion studies have shown that increased FFA levels in

plasma resulted in an increase in intracellular fatty acyl-CoA and diacylglycerol (DAG) concentrations in muscle, which led to the activation of proinflammatory kinase PKC- $\theta$ . However, PKC- $\theta$  deficient mice were protected from fat-induced defects in insulin signaling and glucose transport in skeletal muscle (171). These data have established PKC as an important protein mediating fat-induced insulin resistance in skeletal muscle. Activation of PKC- $\theta$  leads to increased IRS-1 serine phosphorylation, which decreased IRS-1 tyrosine phosphorylation and reduced insulin sensitivity (172). Furthermore, PKC- $\theta$  has been reported to contribute to the activation of JNK and IKK $\beta$  and indirectly inactivate IRS (173).

In addition to these serine/threonine kinase cascades, several other pathways also contribute to inflammation-induced insulin resistance. For example, at least 3 members of the suppressors of cytokine signaling (SOCS) family, SOCS1, -3, and -6, have been implicated in cytokine-mediated inhibition of insulin signaling. SOCS-1 and SOCS-6 were shown to inhibit insulin receptor signaling in adipocytes (174). SOCS-3 expression was found to be increased in the adipose tissue of obese mice. The SOCS-3 mRNA level could be induced by TNF- $\alpha$  in adipose tissue of obese mice, while a pronounced decrease in SOCS-3 expression was observed in the adipose tissue of transgenic ob/ob mice devoid of TNF $\alpha$  signaling (175). SOCS-3 has also been shown to regulate leptin signaling. SOCS3-deficient mice were resistant to high-fat diet-induced obesity, hyperleptinemia and insulin resistance (176, 177). SOCS proteins appear to inhibit insulin signaling by interfering with IRS-1 and IRS-2 tyrosine phosphorylation or by ubiquitin-mediated proteosomal degradation of IRS-1 and IRS-2. Inflammatory cytokine stimulation can also increase the expression of inducible nitric oxide synthase (iNOS). iNOS was increased in muscle and fat of genetic and dietary models of obesity (178, 179). In the insulin signaling pathway, NO can reduce Akt activity by causing s-nitrosylation of a specific cysteine

residue. Increased iNOS activity also results in the degradation of IRS-1 in cultured skeletal muscle cells. Deletion of iNOS prevents impairment of insulin signaling in muscle caused by a high-fat diet (179). Thus, induction of SOCS proteins and iNOS represent two additional and potentially important mechanisms linking cytokine-mediated inflammation to insulin resistance.

### **Chapter 3 Inhibitory effects of stearidonic acid on differentiation of 3T3-L1 adipocytes**

#### **Abstract**

Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from preadipocytes. N-3 PUFAs found in fish oil has been shown to have anti-obesity effects through inhibition of adipocyte differentiation. The aim of this study was to investigate the effect of another n-3 PUFA, SDA, on differentiation of 3T3-L1 adipocytes.

3T3-L1 cells were treated with a differentiation cocktail containing SDA or ethanol vehicle. Adipocyte differentiation was examined by lipid accumulation via triacylglycerol assay and Oil O Red staining. Intracellular triglyceride accumulation in 200  $\mu$ M SDA treated cells was significantly decreased 49% compared to vehicle-treated cells. Calcein-AM/propidium iodide staining was performed to evaluate the effect of SDA on cell viability. SDA-treatment did not affect cell viability. The mRNA level of adipogenic transcription factors and lipid accumulating genes was measured by real-time PCR. On day 3 of differentiation, the expression of the adipogenic transcription factors C/EBP $\beta$ , C/EBP $\alpha$  and PPAR $\gamma$  were significantly decreased from 45% to 73% ( $P < 0.05$ ) in 200  $\mu$ M SDA-treated cells compared to vehicle-treated cells. After treatment for 6 days, 200  $\mu$ M SDA also significantly decreased the lipid accumulation genes aP2, GLUT4, LPL, FAS, and SCD-1 from 59 to 96% ( $P < 0.05$ ). PPAR $\gamma$  binding activity was detected by PPAR $\gamma$  transcription factor assay. PPAR $\gamma$  binding activity was significantly



decreased 25% by SDA treatment on day 3 ( $P < 0.05$ ). In addition, the expression of adipocyte specific proteins was detected by Western blot. The protein expression of adiponectin was also decreased 58% by SDA treatment ( $P < 0.05$ ). Cell proliferation was assessed by  $^3\text{H}$  thymidine incorporation. SDA dramatically reduced 87% of  $^3\text{H}$ -thymidine incorporation on the second cycle of 3T3-L1 cell division ( $P < 0.05$ ).

These results demonstrated that SDA inhibited adipocyte proliferation and suppressed its differentiation into adipocyte. These effects may be caused by the inhibition of the expression of transcriptional factors and lipid accumulation genes and adipocyte specific protein during adipocyte differentiation. SDA may possess anti-obesity effects through prevention of adipogenesis thus making it a possible botanical source of n-3 dietary PUFA in the treatment of obesity.

## **Introduction**

Obesity is a serious health problem in many countries, because it is closely linked to various diseases such as type 2 diabetes, hypertension, coronary heart disease, and cancer (4). Obesity arises from the imbalance between energy intake and energy expenditure, resulting in excess calories being stored as fat in adipose tissue. Although genetic defects have been associated with obesity, habitual food intake in excess of energy expenditure is the primary factor leading to obesity. Dietary n-3 PUFA eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) from fish or fish oil have been shown to protect against the development of obesity and reduce body fat (8, 180-185). However, because many people do not like the taste or smell of fish or fish oils, and due to concerns regarding the safety and

sustainability with fish (9), there is a need to identify and develop alternative sources of (n-3) PUFA.

Stearidonic acid (SDA; 18:4 n-3) has been targeted as a potential biologically active surrogate for EPA, due to its relatively efficient conversion following consumption. SDA represents the  $\Delta 6$  desaturation product of  $\alpha$ -linolenic acid (ALA), and thus bypasses the rate-limiting step in the conversion of dietary ALA to EPA. When SDA enters the metabolic pathway after the  $\Delta 6$  desaturase step, it is rapidly converted to EPA (2). SDA is found in the seeds of several plants, including those of the boraginaceae family, such as *Echium plantagineum*. Consumption of SDA as ethyl esters, Echium Oil, SDA-soybean oil increased EPA levels in plasma, neutrophil, heart, and erythrocyte phospholipids (11-15). Clinical studies indicated dietary SDA as ethyl esters or in SDA soybean oil is 17-30% effective as dietary EPA at raising EPA levels in red blood cell and in plasma phospholipids (12-15). Furthermore, experimental and clinical studies indicated that SDA shares many of the biological effects of n-3 PUFA and functions similarly to dietary EPA (11, 15, 17, 18).

Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue. The entire adipogenic process consists of the preadipocyte proliferation and their differentiation into mature adipocyte, and this proliferation is a prerequisite for adipogenesis. Under receiving the appropriate combination of adipogenic signals, 3T3-L1 preadipocytes proceed through at least two rounds of cell division as mitotic clonal expansion and then initiate the differentiation program (86). The process of adipocyte differentiation is induced by a number of adipogenic transcriptional factors including peroxisome proliferator-activated receptor (PPAR)  $\gamma$  and CCAAT/enhancer-binding protein (C/EBP) family. They are involved in the sequential expression of adipocyte-specific

proteins such as lipoprotein lipase (LPL), adipocyte fatty acid binding protein (aP2), glucose transporter 4 (GLUT4), fatty acid synthase (FAS), and Stearoyl-CoA desaturase 1 (SCD-1), which mediate fatty acid and glucose uptake into adipocytes, triglyceride hydrolysis, and lipogenesis (21, 22), thereby leading to the characteristic phenotype of mature adipocyte. The saturated fatty acid palmitate has been reported to cause a strong stimulation of adipocyte differentiation by increasing both the postconfluent proliferation of preadipocytes and the level of expression of terminal differentiation-related genes (17). In contrast, n-3 PUFA DHA and EPA inhibit adipocyte differentiation (11, 18) and reduce transcription of the differentiation marker gene PPAR $\gamma$  in 3T3-L1 adipocytes (24, 186).

We hypothesize that SDA, as a metabolic surrogate for EPA, has potential anti-adipogenic properties. We determined the effect of SDA on adipocyte differentiation and the expression of adipogenic transcriptional factors and lipid accumulation genes. In addition, we also measured the effect of SDA on adipocyte proliferation.

## **Materials and Methods**

### **Materials**

3T3-L1 mouse fibroblasts were obtained from American Type Culture Collection (ATCC Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), fetal bovine serum (FBS), glutamine, penicillin streptomycin and phosphate-buffered saline (PBS) were obtained from Invitrogen, Inc. (Carlsbad, CA). Isobutylmethylxanthine (IBMX), dexamethasone (DEX), bovine insulin, Oil Red O and anti- $\beta$ -actin antibody was obtained from

Sigma (St. Louis, MO). Anti-GLUT4 antibody was obtained from Cell Signaling (Boston, MA). Anti-adiponectin was obtained from Abcam (Cambridge, UK). Anti-caveolin-1 was obtained from BD Biosciences (San Jose, CA). SDA, ALA, EPA or DHA stock solution in ethanol were purchased from Cayman (Ann Arbor, MI). Bovine serum albumin (BSA), chloroform, methanol, isopropanol were obtained from Fisher Scientific (Fairlawn, NJ). Oligonucleotide primer pairs were ordered from Integrated DNA Technologies, Inc (Coralville, IA). [<sup>3</sup>H] Thymidine was obtained from American Radiolabeled Chemicals, Inc. (St.Louis, MO). All other chemicals were obtained from Sigma.

### **Cell culture**

3T3-L1 cells were differentiated into adipocytes as previously described (187). Briefly, 3T3-L1 cells were cultured in DMEM containing 10% FCS and 1% penicillin–streptomycin at 37 °C and 10% CO<sub>2</sub> until 100% confluent. Two days post-confluence (day 0), the cells were stimulated to differentiation with the differentiation medium (25nM IBMX, 500 μM DEX, 670 nM insulin in DMEM containing 10% FBS) for 3 days (day 3). Cells were then maintained in DMEM containing 10% FBS and insulin for another 3 days (day 6). Fatty acids were dissolved in ethanol and stored in the dark as stock solution at -20°C. Fatty acid-supplemented media was prepared as described previously (188). Briefly, fatty acid solution was incubated with differentiation medium at 37 °C for 1 h before being applied to the cell, allowed the binding of fatty acid to albumin in presence of FBS. 10% FBS in the medium contains albumin at a concentration of about 60μM (provided by Dr. Alyssa Hasty in Vanderbilt University). Solutions were filter-sterilized for use in experiments. Fatty acids or ethanol vehicle control were added with the differentiation medium at the same time as induction of differentiation (day 0) and

cultured according to the differentiation protocol. All fatty acids treatments contained 0.2mmol/L  $\alpha$ -tocopherol (Sigma) to prevent lipid peroxidation.

### **Oil Red O staining**

3T3-L1 adipocytes differentiated for 6 days in the absence or presence of fatty acids were washed with phosphate-buffered saline (PBS, pH 7.4) and then fixed for 1h with 10% formalin in PBS. After a wash with distilled water, cells were stained for 2 h with Oil Red O dye (0.5% in 60% isopropanol). Cells were extensively rinsed with water and dried prior to quantification. Spectrophotometric quantification of the stain was performed by dissolving the stained oil droplets in the cell monolayer with 1ml of isopropanol and then measuring absorbance at 510 nm on a microplate reader using the Bio-Tek spectrophotometer (Winooski, VT). The absorbance was normalized to protein content by the BioRad DC Protein Assay Kit (Bio-Rad, Hercules, CA).

### **Triacylglycerol assay**

3T3-L1 adipocytes differentiated for 6 days in the absence or presence of fatty acid were used for determining triacylglycerol concentration. Cell monolayers were washed with PBS, harvested in PBS buffer containing 10 mM EDTA, pH 7.4. Intracellular triglyceride was extracted by the chloroform-methanol method as described protocol by Schwartz et al (189). Briefly, cell lysates were added to 1.5 ml of methanol in a glass tube and vortex, 625  $\mu$ l of chloroform was then added to the tube followed by adding 1ml of water. After vortex, tubes were incubated at room temperature for 2 h to allow phases to separate, and then 1ml of organic phase (bottom layer) was removed and evaporated with nitrogen. Total triglyceride concentration was

assayed using a serum triglyceride determination kit according to the manufacturer's instructions (Sigma). Triglyceride levels were normalized to protein content.

### **Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted according to the manufacturer's instructions via an RNA extraction kit (Qiagen; Valencia, CA) from 3T3-L1 cells undergoing differentiation in the absence or presence of fatty acid at day 3 and day 6. The RNA concentration and purity were assayed by determining the absorbency of the RNA sample at 260 nm and 280 nm using the DV® 530 Life Science UV/Vis spectrophotometer (Beckman, Fullerton, CA). Complementary DNA (cDNA) was synthesized from 1µg of RNA of each sample according to the manufacturer's protocol using an iScript™ cDNA Synthesis Kit (Bio-Rad). Reverse transcription was performed with sample incubation at 25°C for 5 minutes, followed by 42°C for 30 minutes and then 85°C for 5 minutes. The synthesized cDNA was used immediately for RT-PCR or stored in a -20°C freezer. RT-PCR was performed in 25 µl of iQ™ SYBR® Green Supermix (Bio-Rad) using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). Mouse gene specific primers were designed from Primer Bank (<http://pga.mgh.harvard.edu/primerbank/citation.html>), and constructed by Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). Oligonucleotide sequences of the forward (sense) and reverse (antisense) primers used for amplification are listed in table 1. Reaction mixtures were incubated for an initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 55°C for 10 seconds. The cycle threshold ( $\Delta C_T$ ) method was used to measure relative quantification of the target gene, where values were normalized to the reference gene, 36B4. Fold changes of gene expression were calculated by the  $2^{-\Delta\Delta C_T}$  method (190). The statistical analysis was based on  $\Delta C_T$  values.

## **Western Blot**

The cells were washed with ice-cold PBS buffer and harvested from the culture plate with cell lysis buffer (20mM Tris, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail (1 tablet per 10 ml, Roche Applied Science). The cell lysate was centrifuged at 10,000 g at 4 °C for 10 minutes to remove the insoluble material. The protein concentrations were estimated with Bio-Rad DC Protein Assay Reagent using BSA as a standard. The proteins mixed with sample loading buffer (5% SDS, 5% 2-mercaptoethanol, 62.7 mM Tris-HCl, pH 6.8, 10% glycerol, 0.003% bromphenol blue) were boiled at 95°C for 5 minutes, and then separated in 10% sodium dodecyl sulfatepolyacrylamide (SDS-PAGE) gels. The proteins in the gel were transferred to a nitrocellulose membrane (Amersham Biosciences) by the semi-dry transfer method using the transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol). The membrane was blocked in 5% non-fat dry milk (NFDM) in the Tris Buffered Saline (TBS) with Triton X 100 (TBS-T buffer, 10 mM Tris pH 7.4, 100 mM NaCl, 0.1% Tween-20). The blocked membrane was incubated with specific primary antibodies, and then corresponding secondary antibodies. The membrane was developed using an enhanced chemiluminescent substrate (GE Healthcare, Piscataway, NJ) for 5 minutes. The blots were imaged and analyzed using the UVP Bioimaging System and LabWorks software package (UVP, Upland, CA). The relative expression of proteins was quantified according to the reference bands of  $\beta$ -actin.

## **Thymidine incorporation**

3T3-L1 cells in 6-well plates were induced to differentiate in the absence or presence of fatty acid as described above for 24h, 48h or 72 h. [ $^3\text{H}$ ] Thymidine incorporation was measured as described by Tomlinson et al (191). Briefly, cells were treated with 2 $\mu\text{Ci}$  [ $^3\text{H}$ ] thymidine per

well for 12h before harvesting and then washed once with ice-cold PBS and incubated with ice-cold 5% trichloroacetic acid twice for 15min each at room temperature. The cells were washed with PBS and lysed by 0.5N NaOH/0.5% SDS. The intracellular incorporation of [<sup>3</sup>H]-thymidine was measured by counting the radioactivity in half of the cell lysate with the Packard liquid scintillation analyzer (Meriden, CT). The rest of the lysate was subjected for protein concentration determination.

### **Cell viability assay**

3T3-L1 cells were cultured in 6-well plates to day 0, and were treated with SDA (200 and 400  $\mu$ M) or ethanol vehicle for 24 h or 72 h. Cell viability was determined by the Calcein-AM/propidium iodide (PI) staining kit (Dojindo Molecular Technologies). Briefly, cells were suspended in 200  $\mu$ l PBS containing 1 mM Calcein-AM and 1.5 mM PI. After a 45-min incubation at 37  $^{\circ}$ C, cells were examined immediately using a Canon PowerShot S31S-attached Nikon TS100-F inverted microscope to assess the spatial distribution of the living cells in green (calcein staining) and dead cells in red (PI staining). In each well, at least three different random fields were examined. Live and dead cells in each field were quantified by using NIS Elements Basic Research imaging software, and the percentage of cells with exclusively green fluorescence (interpreted as viable cells) was calculated.

### **PPAR $\gamma$ binding assay**

3T3-L1 cells were treated with or without fatty acids for 24 h, 48 h, or 72 h, after which cells were washed with ice-cold PBS buffer and harvested from the culture plate with buffer A (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) containing protease inhibitor cocktail and PMSF (10 $\mu$ L/mL). The cell lysate was centrifuged at 2500 g for 5 minutes, then



nuclei was resuspended in buffer B (0.4 M NaCl, 25% glycerol) containing protease inhibitor cocktail and PMSF (10  $\mu$ L/mL). After 30 mins at 4°C, lysates were spun for 30mins at 20,000g. The supernatant was collected as nuclear protein fraction. Protein concentrations in the nuclear fraction were determined using the Bio-Rad DC protein assay. PPAR $\gamma$  DNA-binding activity in the nuclear protein was determined by an enzyme-linked immunosorbent assay (ELISA)-based PPAR $\gamma$  transcription factor assay (Cayman) that detects PPAR $\gamma$  bound to PPRE-containing double-stranded DNA sequences. Briefly, standards, quality controls, and nuclear extract samples were added to microtiter strips coated with a consensus dsDNA sequence of PPRE. After incubation overnight at 4°C, the wells were washed, and PPAR $\gamma$  primary antibody was added. After incubation, washing, and addition of horseradish peroxidase (HRP)-conjugated secondary antibody, the developing substrate was added. Finally the stop solution was added and the absorbance was read at 450 nm on a microplate reader (Bio-Tek).

### **Statistical analysis**

The results were expressed as mean  $\pm$  SD. The statistical significance of differences between groups was determined by the Student's *t*-test (two-tailed). Statistically significant differences are defined at the 95% confidence interval.

## **Results**

### **SDA decreased lipid accumulation**

To test the effects of SDA on adipocyte differentiation, 3T3-L1 preadipocytes were stimulated to differentiate into adipocyte with differentiation medium. SDA was added to the

differentiation medium at day 0 and lipid accumulation was determined in adipocytes at day 6. As seen in Fig. 3, micrographs of SDA-treated cells at day 6 demonstrated that SDA suppressed lipid accumulation due to the reduction in the number of observed intracellular lipid droplets (Fig. 3A). These observations of reduced lipid content were confirmed by TG assay (Fig. 3B). Compared to the control cells, the TG accumulation was decreased 26% and 49% ( $P < 0.05$ ) in cultures containing 50 and 200  $\mu\text{M}$  SDA, respectively. We also observed that SDA treatment decreased lipid accumulation by Oil Red O staining (Fig. 4A). The quantification of Oil Red O staining demonstrated that 200  $\mu\text{M}$  SDA significantly decreased 3T3-L1 adipocyte lipid accumulation by 32% (Fig. 4B,  $P < 0.05$ ). Similarly, n-3 PUFA EPA and DHA treatment decreased lipid accumulation by 42% and 57%, respectively ( $P < 0.05$ ). In contrast, ALA showed no apparent effect on lipid accumulation. Taken together, treating 3T3-L1 cells with SDA during induction resulted in inhibition of adipocyte differentiation.

### **The effect of SDA on cell viability**

To determine that the effect of SDA on 3T3-L1 cells described above was not due to alterations in cell viability, 3T3-L1 preadipocytes were treated with 200 or 400  $\mu\text{M}$  SDA for 24 h or 72 h, and cell survival was determined using calcein-AM /PI staining. As shown in Fig. 5, 200 $\mu\text{M}$  SDA treatment was not statistically different from the controls treated for 24 h or 72 h. In contrast, 400  $\mu\text{M}$  SDA significantly decreased cell viability from 92% to 8% and 91% to 5%, after 24 h and 72 h, respectively ( $P < 0.05$ ). These results indicated that the concentration of SDA used (200  $\mu\text{M}$ ) did not affect cell viability, and that the inhibitory effect of SDA on preadipocyte differentiation was not due to cell death.

### **SDA decreased adipogenic transcription factors and lipid accumulation genes**

In order to determine whether SDA inhibited adipocyte differentiation by regulating the expression of adipogenic transcriptional regulators, we examined the effects of SDA on the gene expression of PPAR $\gamma$  and C/EBPs during adipocyte differentiation. As shown in Fig. 6A, the expression of transcription factors PPAR $\gamma$  and C/EBP $\beta$  was decreased 39% and 36%, respectively, in 3T3-L1 cells treated with 50  $\mu$ M SDA for 3 days compared to control cells, and the suppression of expression levels of PPAR $\gamma$  and C/EBP $\beta$  by 200  $\mu$ M SDA reached statistical significant (65% and 45%, respectively,  $P < 0.05$ ). In addition, the expression of C/EBP $\alpha$  was significantly decreased in cells treated with 50  $\mu$ M and 200  $\mu$ M SDA in a dose-dependent manner (50% and 73%, respectively,  $P < 0.05$ ).

PPAR $\gamma$  and C/EBPs mediate the transcription of a group of genes related to fatty acid synthesis, oxidation, transport, storage, or energy expenditure. Next, we determined the mRNA level of PPAR $\gamma$  and C/EBPs target genes in SDA-treated 3T3-L1 cells. 6 days after initiating treatment, 200  $\mu$ M SDA significantly downregulated the mRNA expression of aP2, GLUT4, LPL, SCD-1 and FAS (Fig. 6B,  $P < 0.05$ ) from 59 to 96% compared to vehicle-treated cells. In addition, we found another transcription factor SREBP-1 was markedly decreased (56%,  $P < 0.05$ ) at day 6 by SDA treatment. These results demonstrated that SDA can suppress adipocyte differentiation by decreasing expression of adipogenic genes.

### **SDA inhibited the PPAR $\gamma$ binding activity in 3T3-L1 cells**

PPAR $\gamma$  is a key transcriptional factor in regulating adipocyte differentiation. Having demonstrated the effects of SDA on the mRNA level of PPAR $\gamma$ , we went on to determine whether SDA can inhibit the binding of PPAR $\gamma$  to DNA, using a commercially available transcription factor assay that measures the binding of PPAR $\gamma$  to double stranded DNA probes

containing a PPRE sequence. 3T3-L1 cells were treated with 200  $\mu$ M SDA for 24 h, 48 h, or 72 h, after which nuclear extracts were prepared and subjected to PPAR $\gamma$  binding assay. As shown in Fig. 7A, SDA time-dependently inhibited the PPAR $\gamma$  DNA binding activity in 3T3-L1 cells, and this inhibitory effect reached significant difference at day 3 ( $P < 0.05$ ). To compare the effect of SDA and other n-3 fatty acids on PPAR $\gamma$  binding activity, cells were incubated with 50 or 200 $\mu$ M of ALA, SDA, EPA and DHA for 72 h. As shown in Fig. 7B, the above concentration of ALA had no effect on PPAR $\gamma$  DNA binding activity. However, SDA, EPA and DHA inhibited the PPAR $\gamma$  DNA binding activity in a dose-dependent manner. SDA at a concentration of 200  $\mu$ M significantly reduced 25% of PPAR $\gamma$  DNA binding activity compared to the control ( $P < 0.05$ ). Thus, this result is consistent with the previous data that SDA had an inhibitory effect on the expression of PPAR $\gamma$ . Furthermore, EPA and DHA significantly decreased PPAR $\gamma$  DNA binding activity 39% and 34% respectively ( $P < 0.05$ ). Taken together, these results indicated that SDA has the similar effect with DHA and EPA on inhibiting PPAR $\gamma$  DNA binding activity, and this effect is better than ALA.

### **The effect of SDA on the expression of adipocyte-specific proteins**

Since the adipogenic genes were inhibited by SDA, we examined whether the adipocyte-specific proteins were suppressed in 3T3-L1 adipocytes by SDA treatment. Our Western blot analysis showed SDA significantly decreased 58% of adiponectin protein level compared to the control (Fig. 8,  $P < 0.05$ ). However, the protein level of GLUT4 and Caveolin was not affected by SDA treatment, which is not consistent with the mRNA level of GLUT4.

## Thymidine incorporation

The adipogenic process includes both preadipocyte proliferation and adipocyte differentiation. As an early event of differentiation, 3T3-L1 cells undergo two rounds of cell division under DM induction, and suppression of this process appears to abort adipocyte differentiation. To evaluate the effect of SDA and other n-3 PUFA on preadipocyte proliferation, we determined  $^3\text{H}$ -thymidine incorporation into 3T3-L1 cells.  $^3\text{H}$ -thymidine incorporation assay was used to quantitate DNA synthesis. Two day postconfluence cells were incubated with fatty acids for 24 h to 72 h after the induction of the differentiation medium. As compared with the control cells, there was 4-fold and 3-fold increase of  $^3\text{H}$  thymidine incorporation in DM induced cells at 24 h and 72 h respectively (Fig. 9,  $P < 0.05$ ), which is consistent with two rounds of cell division over that period.  $^3\text{H}$ -thymidine incorporation in 3T3-L1 cells was significantly inhibited by DHA treatment during 24 h post DM-treatment. Whereas, SDA did not have an effect on cell proliferation during that time period. On the other hand, a significant increase in  $^3\text{H}$ -thymidine was observed when preadipocytes were incubated with another n-3 PUFA ALA for 24 h. At 72 h, SDA treatment dramatically reduced about 87% of  $^3\text{H}$ -thymidine incorporation in differentiating 3T3-L1 cells compared with DM-induced control cells, and this inhibitory effect is stronger than DHA (55%,  $P < 0.05$ ) and ALA (32%,  $P < 0.05$ ) treatment. Therefore, the inhibitory effect of SDA on preadipocyte proliferation was observed only between 48 h and 72 h of incubation. These results showed that SDA treatment markedly reduced the second cycle of 3T3-L1 cell division, and suggested that SDA may block adipocyte differentiation through a partial inhibition of 3T3-L1 cell proliferation.

## Discussion

Obesity has become a global health problem due to its association with various diseases, such as type 2 diabetes, cardiovascular disease, and cancer. The prevalence of obesity and obesity-related disease has led to major research interests in the inhibition of adipogenesis. A considerable number of studies have suggested that diets enriched in n-3 LCPUFA DHA and EPA found in fish oil can reduce adipose tissue mass and adipocyte size in rodents (184, 192, 193). However, because of pressure on global fish stocks, interest has turned to the potentially infinite supply of plant-based forms of n-3 fatty acids. In the present study, we found that SDA can serve as a botanical source of n-3 dietary PUFA to inhibit 3T3-L1 adipocyte differentiation.

It has been demonstrated that adipose alteration in tissue mass is related to the size and number of adipocytes. The size of adipocytes increases because of increased storage of triacylglycerols from dietary sources or endogenous lipogenic pathways. Adipocyte number increases as a result of increased proliferation and differentiation of preadipocytes (194). Thus, the amount of adipose tissue mass can be decreased by reducing adipocyte numbers and fat content of adipocytes. Adipogenesis, the development of mature fat cells from preadipocytes, is an intensely studied model of cellular differentiation. Mature adipocytes are larger, round, and filled with large triglyceride droplets, which can be distinguished from fibroblast-like preadipocyte in morphology. In the current study, SDA treatment reduced lipid accumulation, as shown by dose-dependent decreases in intracellular lipid droplet formation. The morphological observations were further confirmed by Oil-O-Red staining and TG assay. In addition, our study also compared the effect of SDA and other n-3 PUFA and on adipocyte differentiation. The results indicated SDA has a similar effect as DHA and EPA in inhibiting lipid accumulation on 3T3-L1 cells, and this effect is better than another n-3 PUFA ALA. This is consistent with

previous studies on the influence of EPA on adipocyte lipid accumulation. It has been reported that TG accumulation was reduced by EPA when 3T3-L1 cells were induced to differentiate by methylisobutylxanthine, dexamethasone and insulin treatment, whereas saturated and monounsaturated fatty acids did not affect differentiation (186). Manickam et al. (2010) (23) also reported that EPA markedly reduced lipid droplet size and total lipid accumulation during adipocyte differentiation. In addition, Lee et al. (2008) (195) demonstrated that lipid accumulation was significantly decreased in mature adipocytes following the 24h treatment of EPA. Therefore, SDA has the similar effect as EPA in reducing lipid accumulation in adipocytes.

The concentration range of the present study was based on EPA or DHA doses used in other in vitro studies (23, 24, 195). We tested the effect of 200  $\mu$ M and 400  $\mu$ M SDA on cell viability during the early differentiation process. The data indicated that the viability of cells was not affected by 200  $\mu$ M SDA as determined by Calcein-AM/PI staining. However, 400  $\mu$ M SDA was toxic during the early stage of differentiation. Cells were floated or had altered morphology and cell viability was decreased dramatically by this high-dose treatment. Based on these observations, we conclude that the suppressive effect of 200  $\mu$ M SDA on adipocyte proliferation and differentiation is independent of non-specific toxicity.

The differentiation of preadipocytes is regulated by a complex network of transcription factors, mainly PPAR $\gamma$  and C/EBP families (92, 196). C/EBP $\beta$  is one of the first transcription factors induced during adipocyte differentiation that is required for mitotic clonal expansion and the expression of downstream transcription factors PPAR $\gamma$  and C/EBP $\alpha$  (197). PPAR $\gamma$  and C/EBP $\alpha$  are the central transcriptional regulators of adipogenesis and are necessary for the synthesis of many adipocyte functional proteins (21, 22). The n-3 LCPUFA, DHA and EPA, have been reported to suppress the differentiation of adipocytes by decreasing the expression of

adipogenic transcription factors and their downstream target genes (23, 195, 198). Consistent with these results, we observed decreases of C/EBP $\beta$  expression by SDA treatment at 3 days in a dose-dependent manner. The present study also indicated that SDA treatment inhibited the expression of PPAR $\gamma$  and C/EBP $\alpha$  on day 3 of differentiation, which demonstrated that SDA inhibited adipogenesis by suppressing the transcriptional factor cascade. Consistently, PPAR $\gamma$  transcription factor binding was also inhibited by SDA and other n-3 PUFA treatments, which supports the inhibitory effect of SDA on the gene expression of PPAR $\gamma$ . In addition, this study showed decreased expression of adipocyte-specific genes aP2, GLUT4, LPL, SCD-1, and FAS after 6 days of treatment of SDA. As these genes are all regulated by PPAR $\gamma$  and C/EBP $\alpha$ , the inhibition effect of SDA on these adipocyte-specific genes may be mediated by inhibition of those two transcription factors. The downstream target genes of PPAR $\gamma$  and C/EBP $\alpha$  are adipocyte-specific genes involved in maintaining adipocyte phenotype. Thus, down-regulation of these genes by SDA treatment would explain the observed negative effects of SDA on lipid accumulation and on adipocyte differentiation.

SREBP is a basic helix-loop-helix transcription factor that regulates expression of enzymes and other proteins involved in cholesterol and fatty acid synthesis (199). SREBP-1 is an adipocyte-associated transcription factor. This factor has been reported to induce the expression of SCD-1 and FAS genes that, in turn, may lead to an increase in fatty acid synthesis and uptake, promoting lipid accumulation within the adipocyte (83, 87, 106). In this study, we found SDA treatment markedly decreased expression of SREBP-1, which is consistent with the decreased expression of FAS and SCD-1. Similar to the effect shown here, Ping Zhang et al. (2008) (70) reported that dietary echium oil, containing 12.5% of SDA, has a similar effect to fish oil in reducing the expression of SREBP-1, SCD-1 and FAS in livers of mice compared to a palm oil



diet.

Adiponectin is exclusively expressed in adipocytes that regulated the metabolism of lipids and glucose. PPAR $\gamma$  is a known transcription factor for the adiponectin gene, with its activation leading to increased adiponectin synthesis (200). Adiponectin gene expression is turned on 2 days after the initiation of adipocyte differentiation and maintained at a relatively high level in mature adipocytes. Bueno et al. (2008) (201) found fatty acids EPA and DHA at 250 $\mu$ M decreased adiponectin gene expression in 3T3-L1 adipocytes. The present study found that adiponectin protein level was decreased in adipocytes whose differentiation was inhibited by SDA treatment, which indicated blockage of adipocyte differentiation reduces adiponectin expression.

For differentiation from preadipocytes into mature adipocytes, proliferation of preadipocytes is a prerequisite. Under the induction of differentiation medium, 3T3-L1 preadipocytes proceed through at least two rounds of cell division as mitotic clonal expansion (MCE) and then initiate the differentiation program. A blockade of MCE will abort the differentiation of 3T3-L1 cell (89-91). In our study, addition of differentiation cocktail stimulated DNA replication that initiated within 24 h and reinitiated between 48 h and 72 h, consistent with two rounds of cell division over that period. Previous studies reported that treatment of 3T3-L1 cells with DHA leads to an inhibition of preadipocyte growth (24). Our findings showed that DHA blocked two rounds of cell division induced by differentiation medium (DM). SDA only exerted an inhibitory effect on DM-induced mitosis of 3T3-L1 cells through the second of two rounds of postconfluence mitosis that occurred between 48 and 72 h, and this inhibitory activity was more prominent than DHA and ALA (76). Consistently, in NIH-3T3 cells, cell proliferation was reported to be significantly inhibited by the addition of SDA, but

slightly stimulated by ALA. In fact, in previous studies it has been shown that DHA or EPA treatment significantly decrease <sup>3</sup>H-thymidine incorporation into DNA in several cancer cell lines (202, 203). This is in good agreement with DNA replication inhibition, and might well explain an impairment of postconfluent mitosis in 3T3-L1, with repercussion on adipogenic differentiation. Similarly, we suggest that SDA may inhibit lipid accumulation at least in part by inhibiting proliferation in postconfluent preadipocytes.

SDA is the  $\Delta 6$ -desaturase product of ALA in the bioconversion of ALA to the long chain n-3 PUFA EPA. With increasing abundance of ALA or SDA, the next metabolite to accumulate is EPA. In humans, the conversion of ALA to EPA is in low amounts (less than 7%) and in even low amounts to DHA (less than 1%) (204). These observations suggest that one or more control point must exist beyond ALA metabolism by delta 6 desaturase. One possible control is a second reaction involving delta 6 desaturase itself, since this enzyme catalyses desaturation of ALA to SDA, as well as 24:5 n-3 to 24:6 n-3 (205). With this perspective in mind, let us turn to a consideration of the conversion of SDA to EPA. Some studies have shown that consumption of SDA as ethyl esters, Echium Oil, or SDA-soybean oil increased EPA levels in plasma, neutrophil, heart, liver and erythrocyte phospholipid (12-15). James et al. (2003) (12) assessed the relative efficiencies of dietary SDA, ALA, and EPA ethyl esters in elevating plasma EPA. Dietary SDA was found to increase plasma EPA between threefold and fourfold more efficiently than comparable levels of ALA and was approximately one-third as effective as dietary EPA. However, none of the dietary test fatty acids resulted in an increase of DHA concentration in the phospholipids of either plasma or erythrocytes. A similar result was shown in a clinical study conducted by the same group. Compared to dietary EPA ethyl esters, dietary SDA in SDA-soybean oil increased red blood cell EPA by about 17% (14). However, the levels of DHA in red

blood cell did not change in any dietary group. By the relative effectiveness of the conversion of dietary SDA to EPA, SDA consumption would be expected to confer the health benefits associated with consumption of EPA, but not DHA.

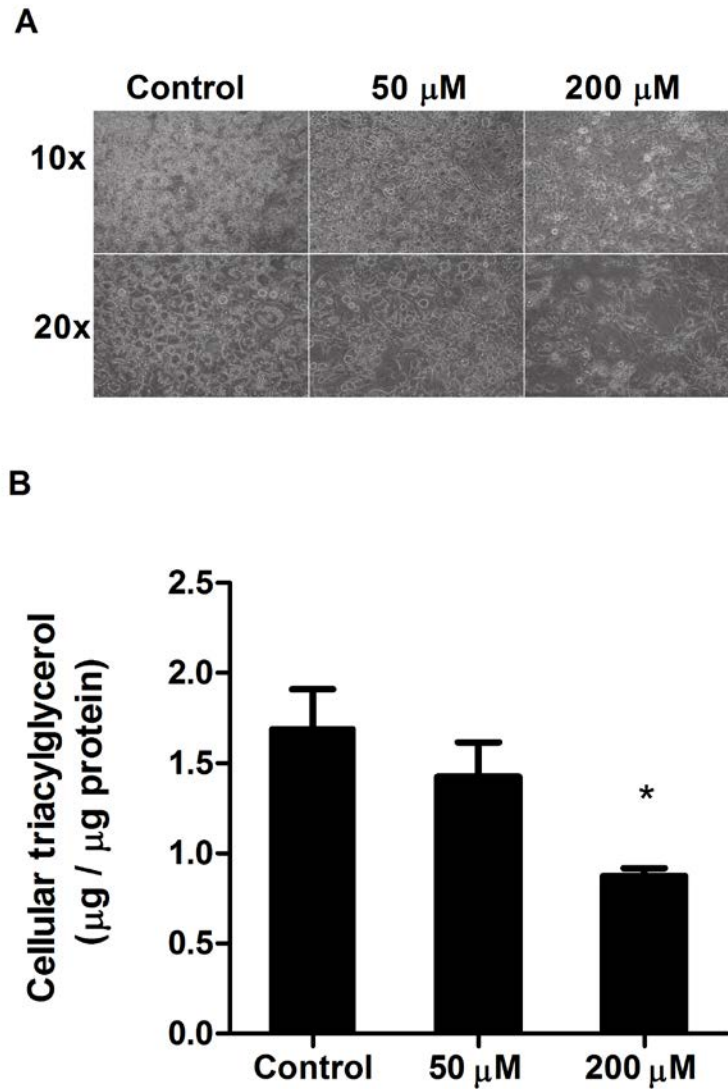
In the present study, we proposed that the effect of SDA on adipocyte differentiation may be caused by its downstream metabolite EPA. The action of EPA was in part due to its incorporation into the sn2 position of membrane phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, which may lead to compelling replacement and/or competition with n-6 PUFA arachidonic acid (AA), resulting in reduced levels of the metabolites of AA, 2-series prostanoids (198, 206, 207). Prostaglandins produced from AA, such as PGI<sub>2</sub> and PGF<sub>2α</sub>, have been reported to be closely related with cell proliferation and terminal differentiation of adipocytes (186). Therefore, the changes of EPA on eicosanoid biosynthesis may be responsible for suppression of adipogenesis. In addition, EPA is also a precursor of resolvins. Although the effect of resolvins on adipocyte differentiation has not been reported previously, it could be a possible mechanism by which EPA suppressed adipocyte differentiation. Thus, in the future study, the influence of SDA on the expression of prostaglandins and resolvins and the direct effect of these metabolites on adipocyte differentiation need to be studied.

In the present study, 3T3-L1 adipocytes were used as a cell culture model to investigate the influence of SDA on adipocyte differentiation. In addition, bovine serum albumin existing in cell culture media containing FBS was used to deliver fatty acid to cells, which is different from the transport of fatty acids by lipoproteins as macromolecules in the blood. To address these problems, the future studies will determine the effect of SDA on adipose tissue mass and morphology in animal model.

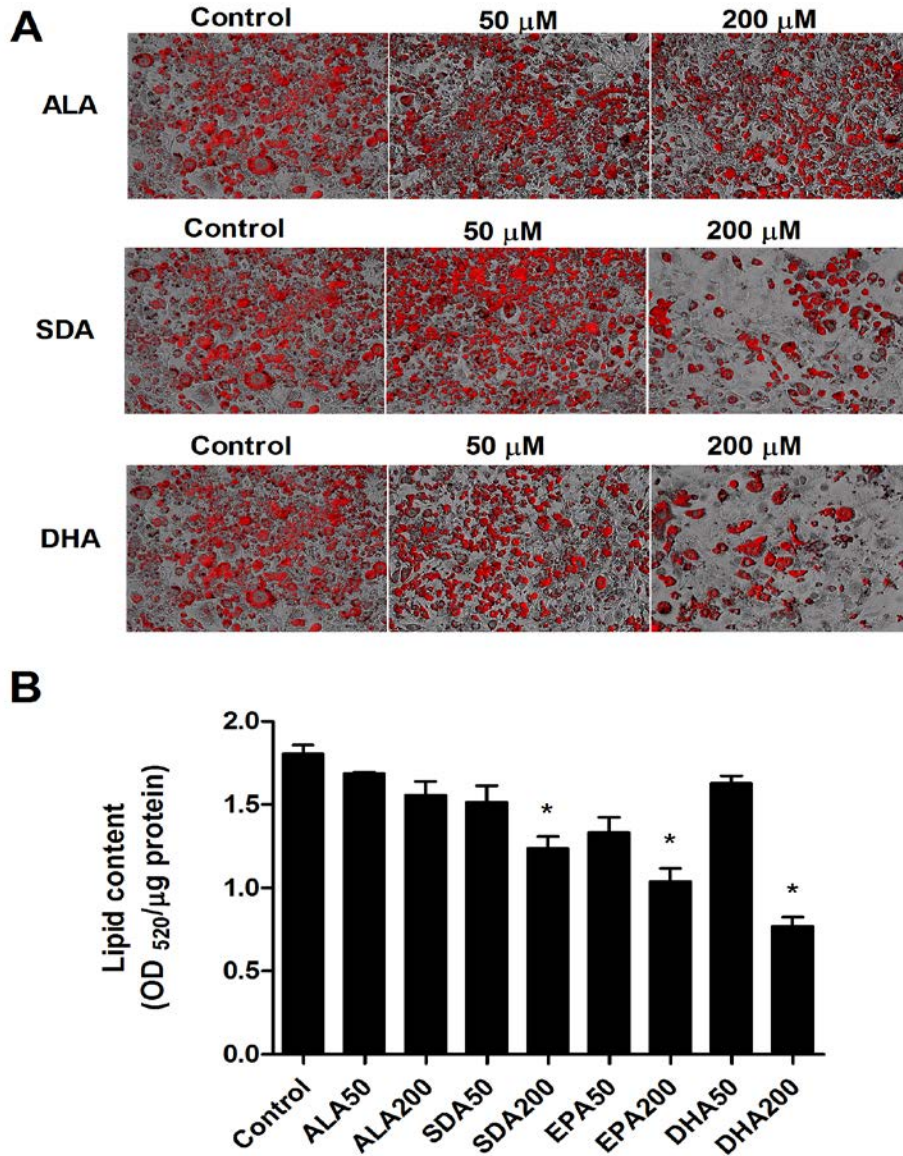
In summary, SDA inhibited 3T3-L1 adipocyte proliferation and differentiation and reduced the lipid accumulation in the adipocyte differentiating process. These effects may work on multiple molecular targets and complex mechanisms including inhibition of mRNA of early transcription factors C/EBP $\beta$ , PPAR $\gamma$ 2, C/EBP $\alpha$  and SREBP-1, and suppression of lipid and glucose accumulation genes SCD-1, FAS, ap2 and GLUT4 and adipocyte specific protein adiponectin. These results were consistent with the previous studies in the inhibition effect of EPA and DHA on adipocyte differentiation. The effects of SDA on the conversion of preadipocyte to adipocyte in adipose tissue remain to be determined in vivo.

**Table 1. Oligonucleotide primer sequences used in RT-PCR for adipogenic transcriptional factors and lipid accumulation genes**

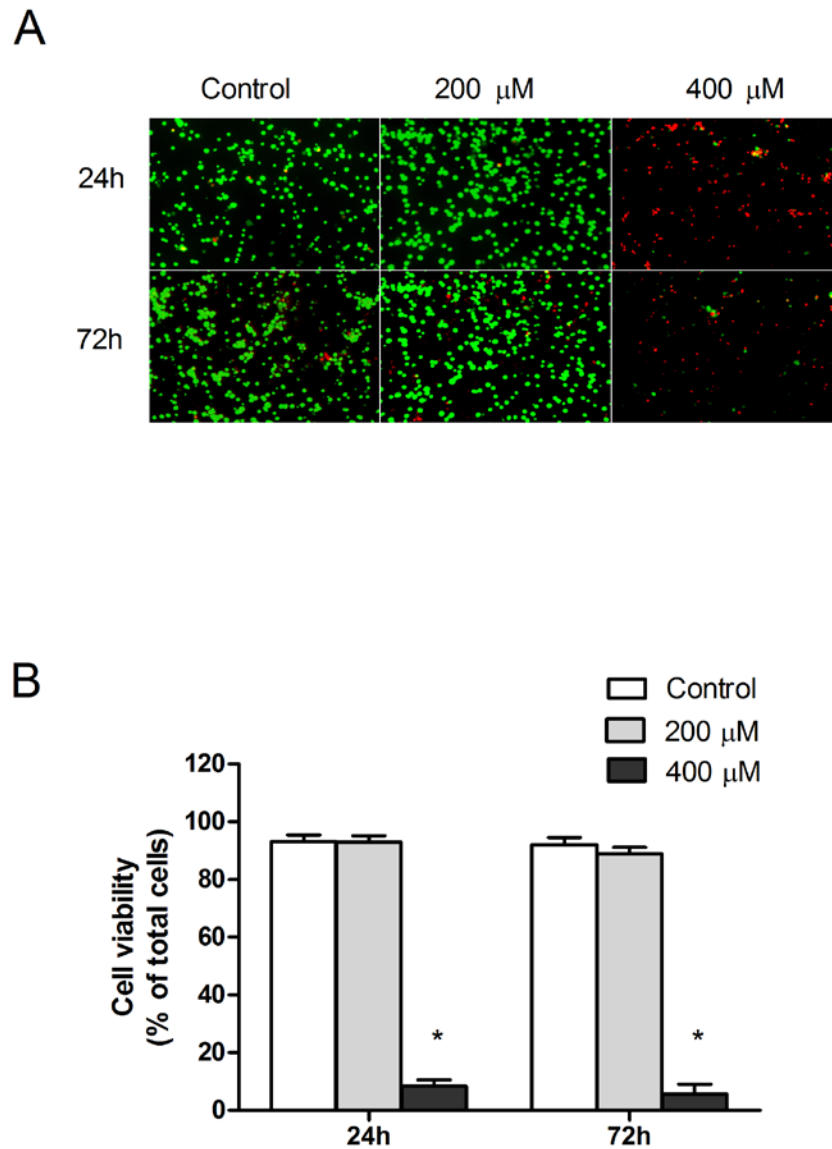
Gene		Sequence
AP2	Forward	5'-AAGGTGAAGAGCATCATAACCCT-3'
	Reverse	5'-TCACGCCTTTCATAACACATTCC-3'
C/EBP $\alpha$	Forward	5'-CAAGAACAGCAACGAGTACCG-3'
	Reverse	5'-GTCACTGGTCAACAGCAC-3'
C/EBP $\beta$	Forward	5' ATGCAATCCGGATCAAACGTGGCT -3'
	Reverse	5'-TTTAAGTGATTACTCAGGGCCCCGGCT-3'
FAS	Forward	5'-GGCTCTATGGATTACCCAAGC -3'
	Reverse	5'-CCAGTGTTTCGTTCCCTCGGA-3'
Glut4	Forward	5'-GTGACTGGAACACTGGTCCTA -3'
	Reverse	5'-CCAGCCACGTTGCATTGTAG-3'
LPL	Forward	5'-GGGAGTTTGGCTCCAGAGTTT -3'
	Reverse	5'-TGTGTCTTCAGGGGTCCTTAG-3'
PPAR $\gamma$	Forward	5'-TGTGGGGATAAAGCATCAGGC -3'
	Reverse	5'-CCGGCAGTTAAGATCACACCTAT-3'
SCD-1	Forward	5'-TTCTTGCGATACACTCTGGTGC-3'
	Reverse	5'-CGGGATTGAATGTTCTTGTCGT-3'
SREBP-1	Forward	5'-GATGTGCGAACTGGACACAG-3'
	Reverse	5'-CATAGGGGGCGTCAAACAG-3'
36B4	Forward	5'-AGA TTC GGG ATA TGC TGT TGG C-3'
	Reverse	5'-TCG GGT CCT AGA CCA GTG TTC -3'



**Fig. 3. The effect of SDA on lipid content of 3T3-L1 cells** Two-day postconfluence cells were induced to differentiate in the presence of SDA (50 or 200  $\mu\text{M}$ ) or ethanol vehicle. (A) Micrographs of control and SDA treated cells at day 6. (B) The levels of triglycerides were measured on day 6. Triglyceride concentration was normalized to cellular protein ( $\mu\text{g TG}/\mu\text{g protein}$ ). Values were obtained from three independent experiments and are expressed as the means  $\pm$  SD; different from ethanol vehicle treated control cells: \* $P < 0.05$

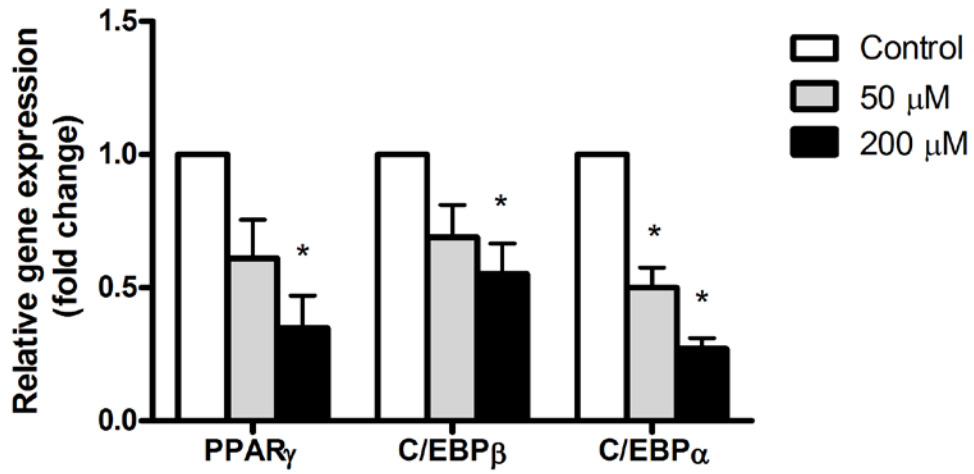
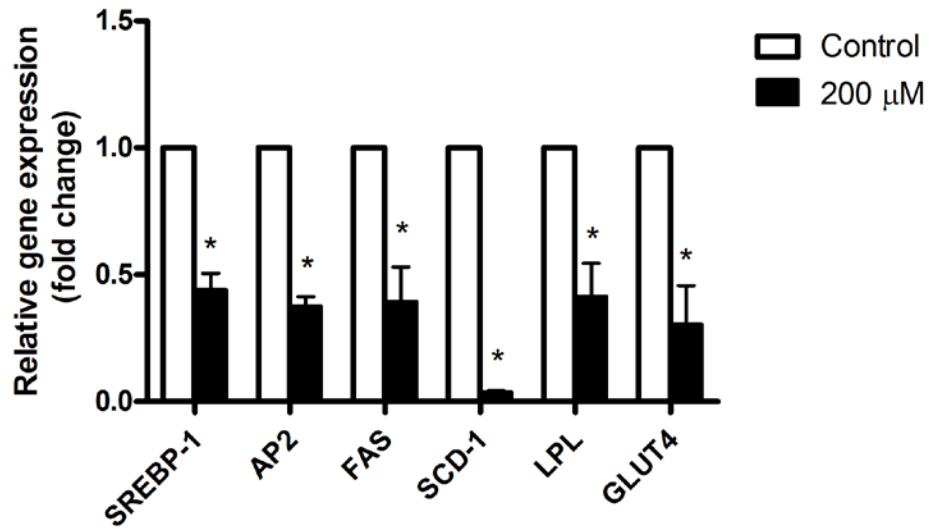


**Fig. 4. The effect of n-3 PUFA on lipid accumulation of 3T3-L1 cells** Two-day postconfluence cells were induced to differentiate in the presence of SDA, ALA, EPA, DHA (50 or 200  $\mu\text{M}$ ) or ethanol vehicle and cultured according to the differentiation protocol for 6 days with treatment. (A) A representative image of Oil Red O staining of cells at day 6. (B) Oil Red O was eluted and quantified at 520 nm. The optical density (OD) was normalized to cellular protein concentration and is given as mean  $\pm$  SD of two independent experiments; different from ethanol vehicle-treated control cells: \* $P < 0.05$



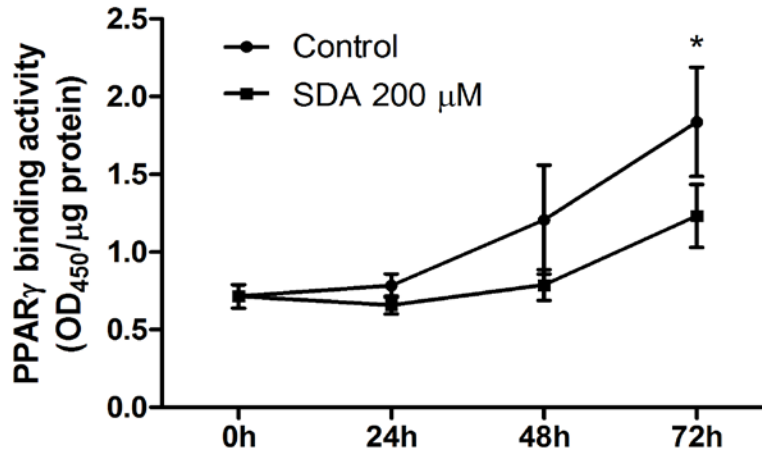
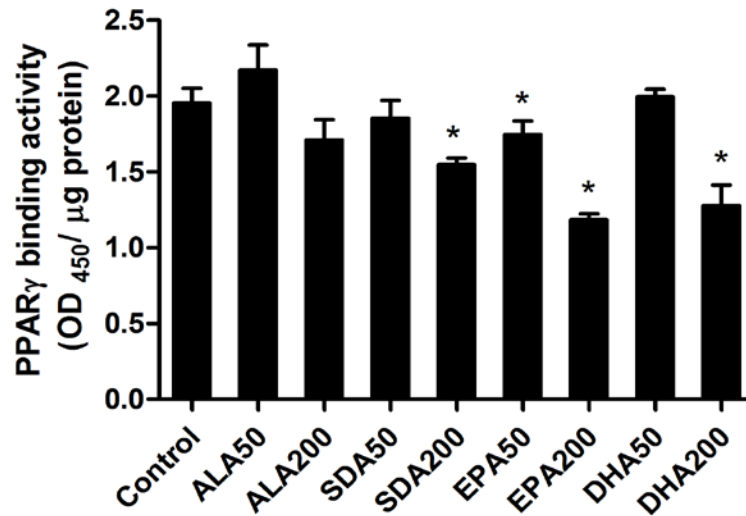
**Fig. 5. The effect of SDA on adipocyte viability.** Two-day postconfluence cells were incubated with differentiation medium containing SDA (200 or 400  $\mu$ M) or ethanol vehicle for 24 h or 72 h. Cell viability was detected by calcein-AM /PI staining. (A) A representative image of cell staining. (B) Quantification of Cell viability. Three images for each treatment were captured and analyzed. Values were obtained from two independent experiments and are expressed as the means  $\pm$  SD; different from ethanol vehicle treated control cells: \*  $P < 0.05$



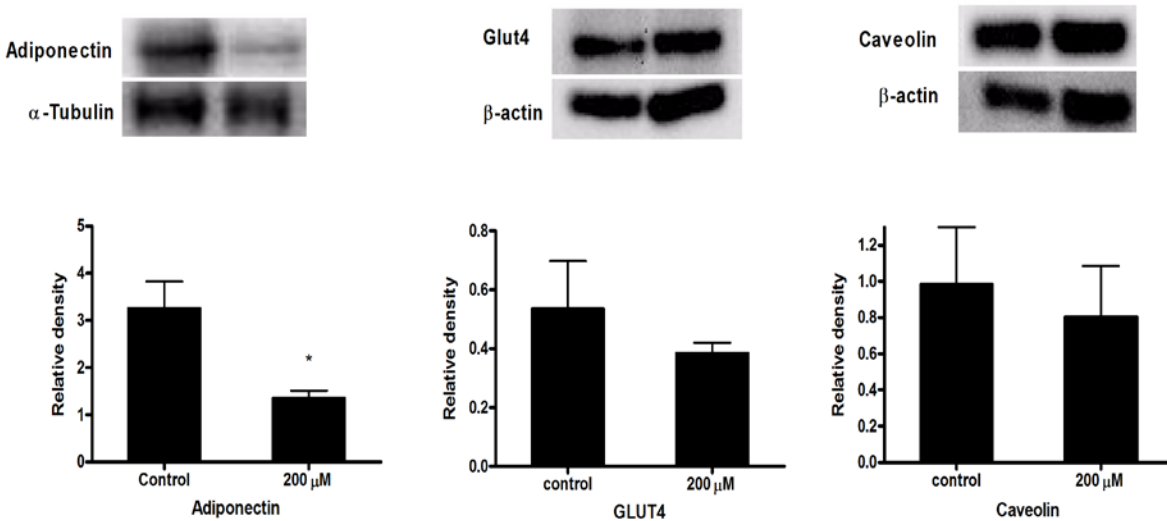
**A****B**

**Fig. 6. The effect of SDA on adipogenic transcription factors and lipid accumulation genes.**

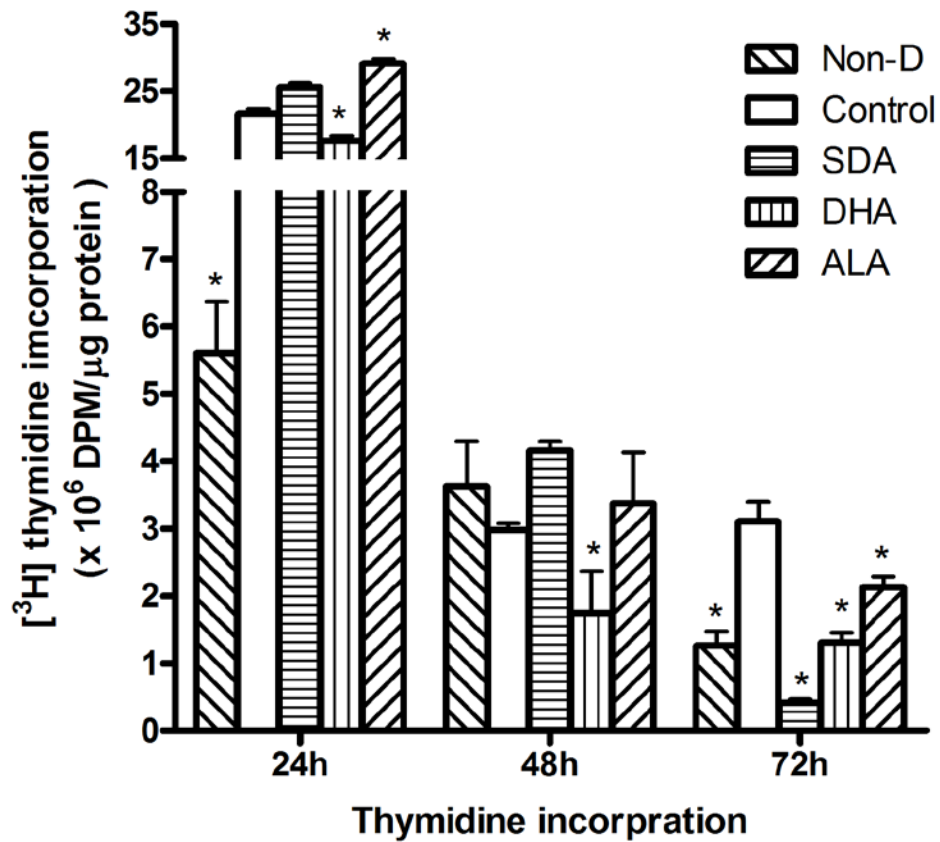
Two-day postconfluence cells were differentiated in the presence of SDA (50 or 200 $\mu$ M) or ethanol vehicle, and adipogenic mark genes were assayed by RT-PCR at day 3 (A) and day 6 (B). Values were normalized to the level of 36B4 mRNA and the fold changes are represented as mean  $\pm$  SD of three independent experiments; different from ethanol vehicle treated control cells: \*  $P < 0.05$  (the statistical analysis was based on  $\Delta$ Ct values).

**A****B**

**Fig.7. The effect of n-3 PUFA on PPAR $\gamma$  binding activity in 3T3-L1 cells.** (A) Two-day postconfluence cells were incubated with differentiation medium containing SDA (200 μM) or ethanol vehicle for 24 h, 48 h, or 72 h. (B) Two-day postconfluence cells were incubated with differentiation medium containing ALA, SDA, EPA, DHA (50 or 200 μM) or vehicle for 72h. DNA binding activity was determined in nuclear extracts of 3T3-L1 cells using a PPAR $\gamma$  transcription factor assay kit. Values are expressed as means  $\pm$  SD; n=3; different from ethanol vehicle treated control cells: \*  $P < 0.05$



**Fig. 8. The effect of SDA on the expression of adipocyte-specific proteins.** Two-day postconfluence cells were incubated with differentiation medium containing 200 μM SDA or ethanol vehicle for 6 days. The protein levels of Adiponectin, GLUT4 and Caveolin were determined by Western Blot analysis. Shown are representative blots. The relative expression of proteins was quantified according to the reference bands of β-actin or α-tubulin. Values were obtained from three or more independent and are expressed as means ± SD; different from ethanol vehicle treated control cells: \*  $P < 0.05$



**Fig. 9. The effect of n-3 PUFA on cell proliferation.** Two-day postconfluence cells were incubated with non-differentiation medium (Non-D), or differentiation medium containing 200  $\mu$ M ALA, SDA, DHA or ethanol vehicle control for 24 h, 48 h, or 72 h. Cell proliferation was determined by [ $^3$ H] thymidine incorporation. Data are presented as total dpm per protein concentration. Values were obtained from two independent experiments performed in duplicate or triplicate and were expressed as the means  $\pm$  SD; different from ethanol vehicle treated control cells: \*  $P < 0.05$

## **Chapter 4 The effects of SDA on inflammation and insulin sensitivity in 3T3-L1 cells**

### **Abstract**

N-3 LCPUFAs, EPA and DHA, have been reported to reduce inflammation and insulin resistance associated with obesity. The aim of this study was to compare the effects of SDA and other n-3 PUFAs on inflammation-related cytokine expression and insulin sensitivity in 3T3-L1 adipocytes, and investigate potential mechanisms by which SDA may prevent inflammation and improve insulin sensitivity.

Inflammation was assessed by measuring the interleukin-6 (IL-6) gene expression in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS) in the presence or absence of SDA, EPA, and ALA. Inflammation in 3T3-L1 cells was assessed by measuring adipocyte expression of IL-6 in adipocytes incubated with LPS-conditioned macrophage medium in the presence or absence of SDA, EPA, and ALA. 3T3-L1 adipocytes incubated with conditioned medium showed dramatically increased transcription of the cytokine gene, IL-6, whereas pretreatment with SDA significantly decreased 63% of IL-6 expression ( $P < 0.05$ ), and EPA and ALA decreased IL-6 expression 41% and 39%, respectively ( $P < 0.05$ ). Insulin sensitivity was assessed by measuring phosphorylation levels of AKT and insulin-stimulated glucose uptake. 2-deoxyglucose uptake assay demonstrated that SDA pretreatment for 24 h stimulated insulin-stimulated glucose uptake by approximately 40% in 3T3-L1 adipocytes ( $P < 0.05$ ). Moreover, Western blot analysis suggested that ALA, SDA, EPA, and DHA treatment increased

the amount of phosphorylated-AKT with maximum increase being attained by 200  $\mu$ M EPA (45%), and DHA and SDA at 200  $\mu$ M exhibited better effect (35% and 38%, respectively) than ALA (20%). Finally, we determined the effect of ALA, SDA, EPA and DHA on PPAR $\gamma$  activity in Human Embryonic Kidney (HEK) 293 cells. We found that SDA at 200  $\mu$ M significantly increased transcription activity 1.8-fold ( $P < 0.05$ ), and EPA and DHA significantly activated the transcriptional reporter 2.6- and 2.7-fold, respectively ( $P < 0.05$ ), but ALA had no apparent effect on PPAR $\gamma$  activity.

Collectively, these data suggest that SDA exhibited similar effects as EPA and DHA and was more effective than ALA at attenuating macrophage-induced inflammation and improving insulin signaling and glucose uptake in 3T3-L1 adipocytes. The effects of SDA on inflammation and insulin sensitivity may be mediated by activation of PPAR $\gamma$ .

## **Introduction**

Obesity is associated with low-grade, chronic inflammation of white adipose tissue characterized by increased production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin-6 (IL-6), monocyte chemotactic protein 1(MCP-1), and inducible nitric oxide synthase (iNOS) (25-27). High concentrations of these proinflammatory adipokines may directly contribute to the development of insulin resistance and diabetes by affecting key cellular functions. For example, chronic overproduction of IL-6 and TNF- $\alpha$  have been reported to cause insulin resistance in adipocytes through activation of several inflammatory pathways such as inhibitor kappaB kinase (IKK) and Jun N-terminal kinase (JNK) that interfere with insulin signaling via serine phosphorylation and subsequent inactivation of insulin receptor substrate

(IRS)-1 (208, 209). In addition, locally secreted chemokines like MCP-1 attract pro-inflammatory macrophages into the adipose tissue where they function to scavenge moribund adipocytes. These tissue macrophages are the main source of pro-inflammatory cytokines that further activate the inflammatory program in neighboring adipocytes, exacerbating inflammation and insulin resistance (210). Long-chain n-3 PUFAs, as found in fish and fish oils, have been shown to exert anti-inflammatory actions by decreasing the production of inflammatory eicosanoids, cytokines, and reactive oxygen species and the expression of adhesion molecules (211).

Dysregulation of free fatty acid metabolism is considered to play a pivotal role in the development of insulin resistance and type 2 diabetes. Fasting whole-body FFA and glycerol release from adipocytes is increased in obese women compared with lean women (32). Physiological elevations of plasma FFA inhibit acutely, as well as chronically, insulin-stimulated glucose uptake in a dose dependent fashion (32). FFA can activate at least three inflammation-associated proteins, including protein kinase C (PKC) delta, IKK, and JNK that are involved in the cross-talk between inflammatory and insulin signaling pathways. Experimental studies conducted in animals have demonstrated that both the amount and type of fatty acids ingested affect insulin action and alter insulin sensitivity in target tissues such as muscle, adipose tissue, and liver (34, 212). Diets high in saturated fats or rich in n-6 fatty acids lead to insulin resistance (33, 34). However, replacement of some of the fat with long-chain n-3 PUFAs prevents the development of insulin resistance caused by high-fat feeding (33, 34).

PPAR $\gamma$  belongs to the nuclear hormone receptor superfamily and regulates gene expression upon heterodimerization with the retinoid X receptor by ligating to peroxisome proliferator response elements (PPREs) in the promoter region of target genes. PPAR $\gamma$  has been known to regulate

adipocyte differentiation, fatty acid storage, and glucose metabolism (213-215). Synthetic PPAR $\gamma$  ligands such as thiazolidinediones (TZD) are insulin-sensitizing drugs used in the treatment of diabetes. In addition, PPAR $\gamma$  has recently been recognized as playing an important role in the inflammatory response through its ability to inhibit the expression of inflammatory cytokines (216). While the true endogenous ligands for PPAR $\gamma$  have not been established with certainty, the receptor can be activated in vitro by polyunsaturated fatty acids and their metabolites (104, 217, 218).

This study was conducted to determine whether SDA can mimic EPA and DHA to exert anti-inflammation and insulin-sensitizing actions. We examined the effect of SDA on macrophage-induced inflammation in adipocytes. We also investigated the effect of SDA on insulin signaling and insulin-stimulated glucose uptake in adipocytes. In addition, we determined the direct effects of SDA on PPAR $\gamma$  activity in HEK293 cells.

## **Materials and Methods**

### **Materials**

3T3-L1 mouse fibroblasts, RAW264.7 macrophage cells and Human Embryonic Kidney (HEK) 293 cells were obtained from American Type Culture Collection (ATCC Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), fetal bovine serum (FBS), glutamine, penicillin streptomycin and Phosphate-buffered saline (PBS) were obtained from Invitrogen, Inc. (Carlsbad, CA). Isobutylmethylxanthine (IBMX), dexamethasone (DEX), and insulin was obtained from Sigma (St. Louis, MO). Anti-phospho-AKT antibody was obtained from Cell Signaling (Boston, MA). Anti-AKT antibody was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Fatty acids SDA, ALA, EPA, or DHA were purchased



from Cayman (Ann Arbor, MI). Bovine serum albumin (BSA) was obtained from Fisher Scientific (Fairlawn, NJ). Oligonucleotide primer pairs were ordered from Integrated DNA Technologies, Inc (Coralville, IA). PPRE X3-TK-Luc, pBABE puro PPAR $\gamma$ 2, PSV sport RXR $\alpha$ , pcDNA- $\beta$  gal, and aP2-luciferase plasmid were obtained from Addgene (Cambridge, MA). [ $^3$ H]-2-deoxyglucose was obtained from Perkin Elmer (Waltham, MA). All other chemicals were obtained from Sigma, except those indicated.

### **Fatty acid-BSA complexes preparation**

Fatty acid dissolved in ethanol was precipitated by the addition of 5M NaOH. The ethanol was then evaporated under nitrogen. The precipitated fatty acids were then resuspended in 0.9% NaCl. After that, an aliquot of 24% (w/v) BSA dissolved in H<sub>2</sub>O was added to the tube. The molar ratio of fatty acids to BSA was 3:1. Fatty acids /BSA complexes were aliquoted and stored at -20°C.

### **Cell culture**

3T3-L1 cells were cultured in DMEM containing 10% FCS and 1% penicillin–streptomycin at 37°C and 5% CO<sub>2</sub> until 100% confluent. Two days post-confluence (day 0), the cells were stimulated to differentiation with the differentiation medium (25 nM IBMX, 500  $\mu$ M DEX, 670 nM insulin in DMEM containing 10% FBS) for 3 days. Then cells were maintained in DMEM containing 10% FBS and insulin for another 3 days. Thereafter, cells were maintained in and refed every 2 days with DMEM containing 10% FBS. Cells were treated with fatty acids at 8-12 days after initiation of the differentiation protocol. Fatty acids were delivered to the cells as fatty acid/ bovine serum albumin (BSA) complexes. BSA vehicle was used a control.

RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and incubated at 37 °C in 10% CO<sub>2</sub> until confluence. Cells were treated with fatty acids overnight, followed by incubation with LPS for 8h. The gene expression of IL-6 was assayed by RT-PCR. For the preparation of conditioned medium (CM), RAW264.7 cells were stimulated with LPS (100 ng/mL) for 24h in serum-free DMEM. The media was collected and stored at -80 °C. Immediately before use in the treatment of adipocytes, the medium was diluted with 10% FBS DMEM at the ratio of 1:1 (v/v). Adipocytes in the blank group were treated with DMEM containing 5% FBS.

### **RT-PCR**

Total RNA was extracted from macrophages or adipocytes according to the manufacturer of the RNA extraction kit from Qiagen (Valencia, CA). The RNA concentration and purity were assayed by determining the absorbency of the RNA sample at 260 nm and 280 nm using the DV® 530 Life Science UV/Vis spectrophotometer (Beckman, Fullerton, CA). Complementary DNA (cDNA) was synthesized from 1µg of RNA of each sample according to the manufacturer's protocol using an iScript™ cDNA Synthesis Kit (Bio-Rad). Reverse transcription was performed with the sample incubation at 25°C for 5 minutes, followed by 42°C for 30 minutes and then 85°C for 5 minutes. The synthesized cDNA was used immediately for RT-PCR or stored in a -20°C freezer. RT-PCR was performed in 25 µl of iQ™ SYBR® Green Supermix (Bio-Rad) using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). Mouse gene specific primers were designed from Primer Bank (<http://pga.mgh.harvard.edu/primerbank/citation.html>), and constructed by Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). Oligonucleotide sequences of the forward (sense) and reverse (antisense) primers used for amplification are listed in table 2. Reaction mixtures

were incubated for an initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 55°C for 10 seconds. The cycle threshold ( $\Delta C_T$ ) method was used to measure relative quantification of the target gene, where values were normalized to the reference gene, 36B4 or  $\beta$ -actin. Fold changes of gene expression were calculated by the  $2^{-\Delta\Delta C_T}$  method (190).

## **Western Blot**

The cells were washed with the ice-cold PBS buffer and harvested from the culture plate with cell lysis buffer (20mM Tris, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail (1 tablet per 10 ml, Roche Applied Science). The cell lysate was centrifuged at 10,000 g at 4°C for 10 minutes to remove the insoluble material. The protein concentrations were estimated with the Bio-Rad DC Protein Assay Reagent using BSA as a standard. The proteins mixed with sample loading buffer (5% SDS, 5% 2-mercaptoethanol, 62.7 mM Tris-HCl, pH 6.8, 10% glycerol, 0.003% bromphenol blue) were boiled at 95°C for 5 minutes and then separated in 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. The proteins in the gel were transferred to a nitrocellulose membrane (Amersham Biosciences) by the semi-dry transfer method using the transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol). The membrane was blocked in 5% non-fat dry milk (NFDM) in the Tris Buffered Saline (TBS) with Triton X 100 (TBS-T buffer, 10 mM Tris pH 7.4, 100 mM NaCl, 0.1% Tween-20). The blocked membrane was incubated with specific primary antibodies, and then corresponding secondary antibodies. The membrane was developed using an enhanced chemiluminescent substrate (GE Healthcare, Piscataway, NJ) for 5 minutes. The blots were imaged and analyzed using the UVP Bioimaging System and LabWorks software package (UVP, Upland, CA).

## **2-Deoxyglucose uptake assay**

3T3-L1 adipocytes at 8-12 days after differentiation were used for glucose uptake assay. Basal and insulin-stimulated glucose uptakes were measured as described previously (219). Briefly, serum-starved adipocytes were washed and incubated in Kreb's Ringer phosphate HEPES (KRPB) buffer (136 mM NaCl, 20 mM HEPES, 5 mM sodium phosphate buffer, 4.7 mM KCl, 1 mM MgSO<sub>4</sub>, 1mM CaCl<sub>2</sub>, pH 7.4) containing 0.5% BSA for 15 minutes, and then cells were incubated with or without insulin (100 nM) for 30 minutes. Glucose uptake was determined by the addition of [<sup>3</sup>H]-2-deoxyglucose (2-DOG) (final concentration, 20μCi/ml) for 10 minutes. The reaction was stopped by washing the cells three times with the ice-cold PBS buffer. Cells were then air dried for 15 min and solublized in 0.2 N NaOH. Finally, the intracellular incorporation of [<sup>3</sup>H]-2-deoxyglucose was measured by counting the radioactivity in the cell lysate with the Packard liquid scintillation analyzer (Meriden, CT). Results were normalized for protein content measured by BCA assay.

## **Luciferase assay**

HEK293 cells were cultured in 12-well plates and in complete DMEM media containing 10% FBS and penicillin-streptomycin as antibiotics. After reaching 80% confluence, cells were washed with PBS and transfected using lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instruction. For assessment of PPAR $\gamma$  activity, cells were transfected with 250ng of PPRE X3-TK-Luc, 250ng of pBABE puro PPAR $\gamma$ 2, 50ng of PSV sport RXR $\alpha$ , and 200ng of pcDNA- $\beta$  gal plasmid. For transactivity of aP2, cells were transfected with 250ng of pBABE puro PPAR $\gamma$ 2, 50ng of PSV sport RXR $\alpha$ , 400ng of aP2-luciferase plasmid, and 200ng of pcDNA- $\beta$  gal plasmid. Transfected cells were then incubated at 37°C. Media was changed to

complete DMEM after 4 hours of incubation followed by the overnight incubation. Twenty-four hours after the transfection, the cells were treated with fatty acids or rosiglitazone (as a positive control) for another 24 h. Fatty acid was delivered to the cells as fatty acid/bovine serum albumin (BSA) complexes. The molar ratio of fatty acid to BSA was 3:1. The luciferase activity in the cells was determined using a luciferase assay kit (Promega) in a GLOMax 96microplate luminometer (promega).  $\beta$ -Galactosidase activity was measured using the  $\beta$ -Galactosidase Enzyme Assay kit (Promega) in the Synergy HT Multi-Mode Microplate Reader (Bio-TEK). Luciferase activities were normalized to  $\beta$ -Galactosidase activity and were presented as the fold change (the percentage) of luciferase activity measured in the presence of stimulus relative to the activity of control cells with no stimulation. The luciferase assay experiments were conducted in Dr. Amin's Lab in Pharmacal School of Auburn University.

### **Statistical analysis**

The results are expressed as mean  $\pm$  SD. The statistical significance of differences between groups was determined by the Student's *t*-test (two-tailed). Statistically significant differences are defined at the 95% confidence interval.

### **Results**

#### **SDA and DHA decreased inflammatory cytokine expression in LPS incubated macrophage cells**

LPS is the major component of the outer membrane of Gram-negative bacteria, acts as endotoxins and elicits strong immune responses in animals. A preliminary time course study

showed that 100 ng of LPS treatment for 8 h robustly increased the expression of inflammatory gene IL-6 in macrophage cells. Next, we examined the effect of the pretreatment of SDA on LPS- induced inflammation on macrophage cells. RAW 264.7 cells were pretreated with 50uM SDA (Fig. 10 A) overnight, followed by incubation with 100ng of LPS for 8h. The expression of inflammatory cytokine IL-6 was assayed by RT-PCR. LPS stimulation dramatically increased mRNA expression of IL-6. Pretreatment of RAW264.7 cells with SDA significantly inhibited LPS-induced increases in the expression of IL-6 by nearly 70%. As seen in Fig10. B, a similar result was obtained in RAW264.7 cells pretreated with 200  $\mu$ M DHA. DHA also showed an inhibitory effect on LPS-induced IL-6 expression.

### **N-3 PUFA decreased CM-induced inflammatory cytokine expression in 3T3-L1 cells**

Since macrophage infiltration contributes substantially to adipose tissue inflammation, we next investigated the effects of n-3 PUFA on inflammatory cytokine expression in adipocytes exposed to the macrophage-conditioned medium. We pretreated adipocytes with various fatty acids or a vehicle and then incubated them with conditioned medium from macrophages. As seen in Fig. 11, compared to the blank group, pretreatment with EPA or ALA significantly decreased 63% and 50% of IL-6 expression in 3T3-L1 cells ( $P < 0.05$ ), but SDA showed no effect on that. The addition of the macrophage derived condition medium greatly stimulated the mRNA level of the inflammatory gene IL-6 in adipocytes. Pretreatment of adipocytes with SDA, EPA or ALA attenuated the expression levels of IL-6 induced by condition medium. IL-6 expression levels were suppressed by SDA to a greater extent (63%,  $P < 0.05$ ) than EPA and ALA (41% and 39%, respectively,  $P < 0.05$ ). These results indicate that SDA pretreatment blocked the macrophage conditioned medium-induced inflammation in adipocytes.

### **SDA improved insulin-stimulated glucose uptake in 3T3-L1 cells**

We examined whether SDA could affect the activity of insulin-stimulated glucose transport in adipocyte. Fully differentiated 3T3-L1 adipocytes were incubated with 200  $\mu$ M SDA for 24 h at 37°C, and the basal and insulin-stimulated glucose uptake were assessed by measuring intracellular incorporation of [<sup>3</sup>H]-2 deoxyglucose. As seen in Figure 12, insulin stimulation significantly increased almost 2- fold of the basal 2-deoxyglucose uptake in 3T3-L1 cells. SDA pretreatment for 24 h further increased insulin-stimulated glucose uptake by about 40% ( $P < 0.05$ ). To measure the acute effect of SDA on glucose uptake in adipocytes, cells were pretreated with SDA for 30 min, then the basal and insulin-stimulated glucose uptake were determined. However, a 30 min exposure of adipocytes to the n-3 unsaturated fatty acids SDA could not improve insulin-stimulated glucose uptake as shown in Fig 13.

### **Effects of n-3 PUFA on phosphorylation of AKT in 3T3-L1 cells**

AKT is an important molecule for insulin signaling and glucose metabolism. We already showed that SDA increased insulin stimulated glucose uptake, next we assessed the effects of n-3 PUFA on the activity of AKT by measuring its phosphorylation levels. Western blot analysis indicated that the phosphorylation of AKT began to increase after the adipocytes had been treated with SDA, EPA, and DHA for 15 min and the highest level was observed after 30-60 min of fatty acid treatment (Fig. 14 A). Furthermore, we treated adipocytes with n-3 fatty acids at different dose for 30 min. As shown in Fig. 14 B, ALA, SDA, EPA, and DHA treatment showed an increasing trend in the amount of phosphorylated-AKT in a dose-dependent manner with maximum increase being attained by 200  $\mu$ M EPA (45%). DHA and SDA at 200  $\mu$ M increased the level of phosphorylated-AKT by 35% and 38%, respectively, whereas ALA treatment at that

concentration only showed 20% increase. However, these results were not statistically significant.

### **Effect of fatty acids on PPAR $\gamma$ activity**

Given the important role that PPAR $\gamma$  plays in regulating inflammation and insulin resistance, we next examined the effect of SDA or other fatty acids on PPAR $\gamma$  activity. HEK293 cells were transfected with the PPRE reporter in combination with an expression plasmid encoding PPAR $\gamma$ . Following transfection, cells were treated with 50 and 200  $\mu$ M ALA, SDA, EPA, DHA, Oleic acid (OA; n-9 18:1), 10  $\mu$ M rosiglitazone, or the vehicle control. As shown in Fig. 15, the addition of rosiglitazone, a TZD ligand for PPAR $\gamma$ , stimulated a 3.7 fold increase of transcriptional activity obtained from PPAR $\gamma$  when compared with vehicle control cell. Treatment with 200  $\mu$ M ALA only slightly enhanced PPAR $\gamma$  activation 1.3 fold. However, SDA at 200  $\mu$ M significantly increased the transcription activity 1.8 fold ( $P < 0.05$ ). EPA at 50 and 200  $\mu$ M significantly increased PPAR $\gamma$  activity 1.5 and 2.6, fold respectively ( $P < 0.05$ ). Furthermore, DHA at 50 and 200  $\mu$ M activated the transcriptional reporter by 1.7 and 2.7 fold, respectively ( $P < 0.05$ ). Thus, the effect of fatty acids ALA, SDA, EPA, and DHA on PPAR $\gamma$  activity increased with the degree of unsaturation (DHA>EPA>SDA>ALA). On the other hand, oleic acid at 50 and 200  $\mu$ M significantly decreased the activation of PPAR $\gamma$  by 47% and 30%, respectively ( $P < 0.05$ ).

### **Effect of fatty acids on aP2 activity**

Since aP2 also mediates PPAR-dependent transactivation, we next determined whether SDA and other n-3 PUFA have an effect on the transcriptional activity of aP2. HEK293 cells were transfected with PPAR $\gamma$ , RXR $\alpha$ , along with a reporter construct containing two PPREs



derived from the aP2 gene enhancer (aP2-Luc plasmid). Following transfection, the cells were treated with Rosi or n-3 fatty acids. As shown in Fig. 16, Rosi caused an ~2-fold increase in aP2 promoter-luciferase activity, which was mediated by co-transfected PPAR $\gamma$ . However, in the same cells, ALA, SDA, and EPA had no effect on PPAR $\gamma$ -mediated aP2 transcription. Only DHA significantly increased aP2 promoter-luciferase activity 1.3 fold ( $P < 0.05$ ).

## Discussion

Obesity is associated with a chronic, low-grade inflammation characterized by inflamed adipose tissue with increased infiltration of macrophages that produce pro-inflammatory cytokines such as IL-6 and TNF $\alpha$ . Proinflammatory factors secreted from macrophages not only induce adipose tissue inflammation, but also impair insulin signaling, leading to insulin resistance. Previous studies showed that a diet rich in saturated and monounsaturated fatty acids leads to extensive adipose tissue infiltration with macrophages together with increased expression with a number of inflammatory genes in obese diabetic mice (220). However, inclusion of n-3 LCPUFA EPA and DHA in the diet completely prevented macrophage infiltration of adipose tissue and changes in inflammatory gene expression induced by a high-fat diet in obese diabetic mice (220). In another study, Hsueh et al. (2011) (221) showed that SDA and EPA inhibit LPS-induced IL-6 expression in adipose stem cells by decreasing the TLR2-mediated signaling pathway. Consistently, our data indicated that SDA exerted a similar effect with DHA on decreasing LPS-induced IL-6 gene expression in macrophage cells. Furthermore, pretreatment with SDA, EPA, or ALA attenuated the expression levels of IL-6 induced by the macrophage derived conditioned medium. IL-6 expression level was suppressed by SDA to a

greater extent than EPA and ALA. The direct anti-inflammatory effects of SDA on macrophages and adipocytes demonstrated in this study suggest that long-term treatment with SDA may mimic EPA or DHA to reduce proinflammatory macrophage infiltration into adipose tissue, which needs to be further investigated *in vivo*.

Chronic, low grade tissue inflammation is an important cause of obesity-related insulin resistance. Oh et al. (2010) (222) reported that n-3 fatty acids DHA and EPA mediated anti-inflammatory effects in proinflammatory macrophages and mature adipocytes by signaling through the G protein-coupled receptor 120 (GPR120). This study also showed that the anti-inflammatory actions of n-3 fatty acids were coupled to insulin sensitizing effects in wild type mice, but not in GPR120 knockout mice (222). In the present study, we already showed that SDA has a similar anti-inflammatory effect with DHA or EPA. We then examined whether SDA could affect insulin sensitizing by measuring insulin-stimulated glucose transport in adipocyte. Controversial results regarding the effects of n-3 PUFA on insulin-stimulated glucose uptake have been previously reported. Nugent et al. (2001) (223) have shown that 4-8 h exposure of 3T3-L1 cultures to a selection of unsaturated fatty acids ranging from 16:1 to 22:6 resulted in increased insulin stimulated glucose uptake. In this study, when adipocytes were pretreated with SDA for 24 h, SDA had no effect on basal glucose uptake, but significantly increased insulin-stimulated glucose uptake. This differs from the tendency reported by Perez-Matute et al. (2005) (224), 96 h treatment of EPA increased basal glucose utilization, but had no effect on insulin-stimulated glucose uptake in isolated rat adipocytes. But this may be due to differences in cell type and time of exposure to fatty acids. Oh et al. (2010) reported that 30 min pretreatment with DHA significantly increased both basal and insulin-stimulated glucose uptake in 3T3-L1 cells (222). In our study, short-term exposure of adipocytes to SDA could not improve insulin-

stimulated glucose uptake. These results are in agreement with the report by Aas et al. (225) in which a 30 min exposure of adipocytes to the unsaturated fatty acids OA, arachidonic acid (AA; n-6 20:4), EPA, and DHA could not improve insulin-stimulated glucose uptake in isolated rat epididymal adipocytes.

Since AKT is involved in the pathway for insulin-stimulated GLUT4 translocation and glucose uptake, and our data indicated that SDA increased insulin-stimulated glucose uptake. Next we determined the activity of AKT by measuring its phosphorylation levels in 3T3-L1 adipocytes. In our study, SDA, EPA, and DHA treatment for 30 min showed an increase trend in the amount of phosphorylated-AKT in a dose-dependent manner, and less effect was observed by ALA treatment. This result is consistent with a previous study that showed 30 min treatment with DHA had a modest effect to stimulate the phosphorylation of AKT on differentiated 3T3-L1 cells (222).

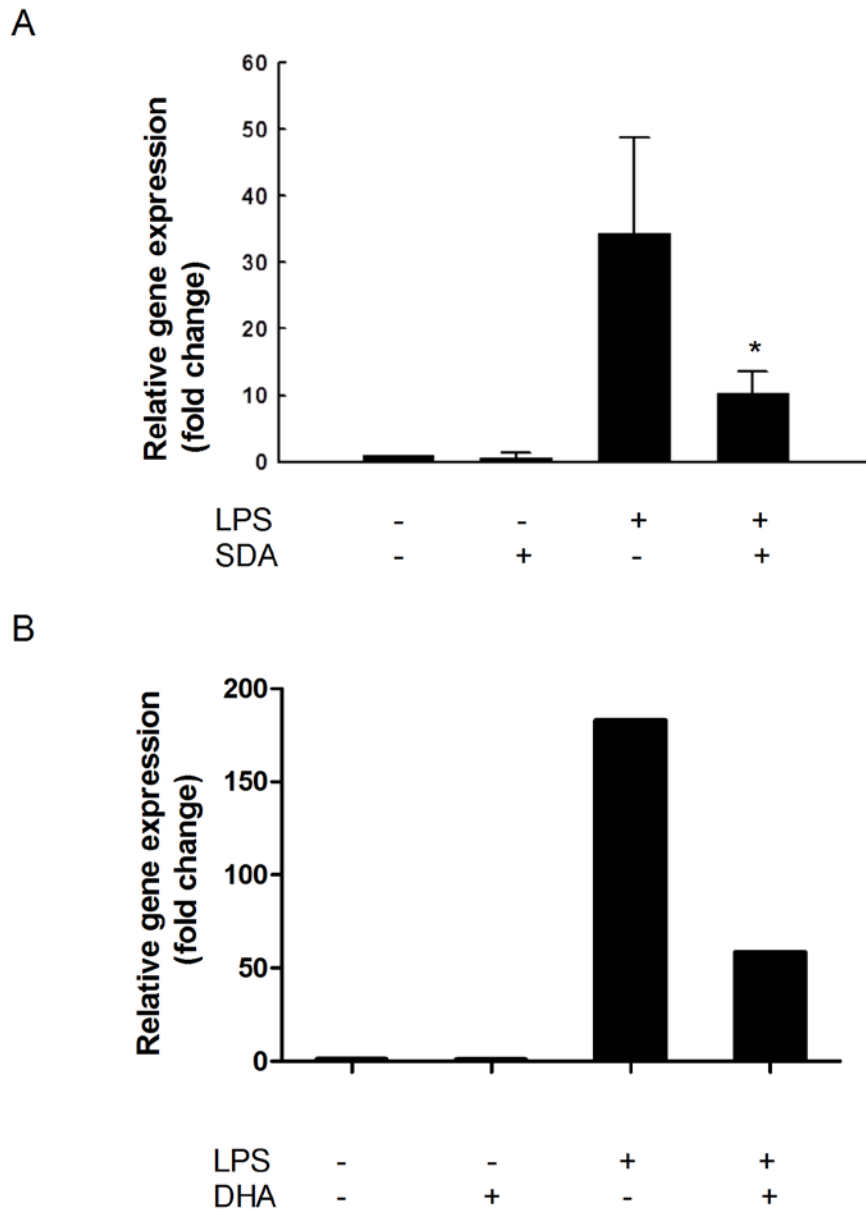
PPAR $\gamma$  is essential for adipocyte proliferation and differentiation. In recent years, PPAR $\gamma$  and its ligands, the TZD, have achieved great attention due to their insulin sensitizing and anti-inflammatory properties. Pharmacological agonists for PPAR $\gamma$  can enhance glucose disposal (94, 226, 227), and loss of function mutations in human PPAR $\gamma$  result in insulin resistance and diabetes mellitus (228). N-3 PUFAs DHA and EPA and their metabolisms have been recognized as nature ligands for PPAR $\gamma$  (97, 229-231). Consistent with previous studies, our study showed that EPA and DHA increased PPAR $\gamma$  transactivation in a dose-dependent manner when PPAR $\gamma$  was overexpressed in HEK293 cells by transient transfection. We also showed that SDA had the similar effect with EPA and DHA on stimulating PPAR $\gamma$  activity, but another n-3 fatty acid ALA had no apparent effect on PPAR $\gamma$  activation. Interestingly, in our study, the progressive desaturation of the fatty acid molecule led to a progressive increase of

PPAR $\gamma$  transactivation (DHA>EPA>SDA>ALA). In contrast, the monounsaturated fatty acid oleic acid significantly decreased the activation of PPAR $\gamma$ . Indeed, PPAR $\gamma$  agonists such as TZD reduce insulin resistance or inflammation associated with obesity. In our study, we showed that SDA has potential anti-inflammatory and insulin-sensitizing effects in adipocytes. Thus, we proposed that SDA may attenuate inflammation and stimulate glucose uptake by regulating PPAR $\gamma$  activity. Since aP2 mediates PPAR-dependent transactivation, we also determined whether SDA and other n-3 PUFA have an effect on the transcriptional activity of aP2 in HEK293 cells. DHA caused a 1.3-fold increase in aP2 promoter-luciferase activity, which was mediated by co-transfected PPAR $\gamma$ . However, ALA, SDA, and EPA had no effect on PPAR $\gamma$ -mediated aP2 transcription. Since this reporter only contains two PPREs derived from the aP2 gene enhancer, and PPAR $\gamma$  agonists Rosi caused less effect (~2-fold increase) on aP2 promoter-luciferase activity than on PPAR $\gamma$  activity (3.7-fold increase). This may explain why EPA and SDA can increase PPAR $\gamma$  activity, but had no effect on aP2 promoter-luciferase activity.

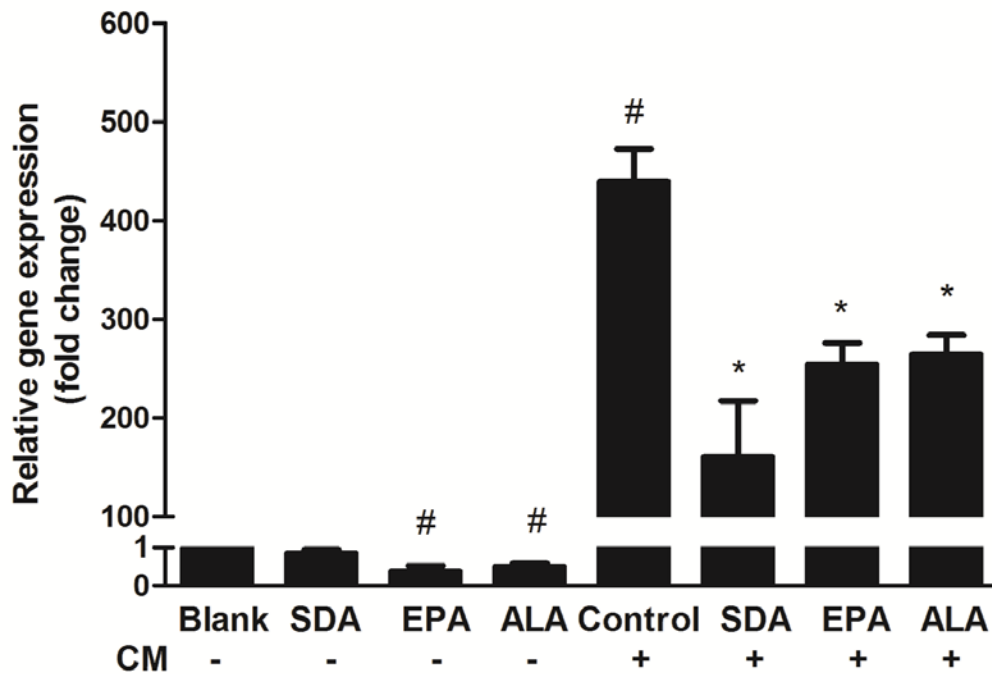
In summary, this study showed that SDA attenuated inflammation-related cytokine and improved insulin signaling and insulin-sensitizing in 3T3-L1 adipocyte. In addition, our study also demonstrated that SDA, EPA and DHA can increase PPAR $\gamma$  transactivity. Thus, we propose that the beneficial effects of SDA and other n-3 PUFAs on inflammation and insulin sensitivity may be mediated by activation of PPAR $\gamma$ .

**Table 2. Oligonucleotide primer sequences used in RT-PCR for IL-6, 36B4, and  $\beta$ -actin gene expression**

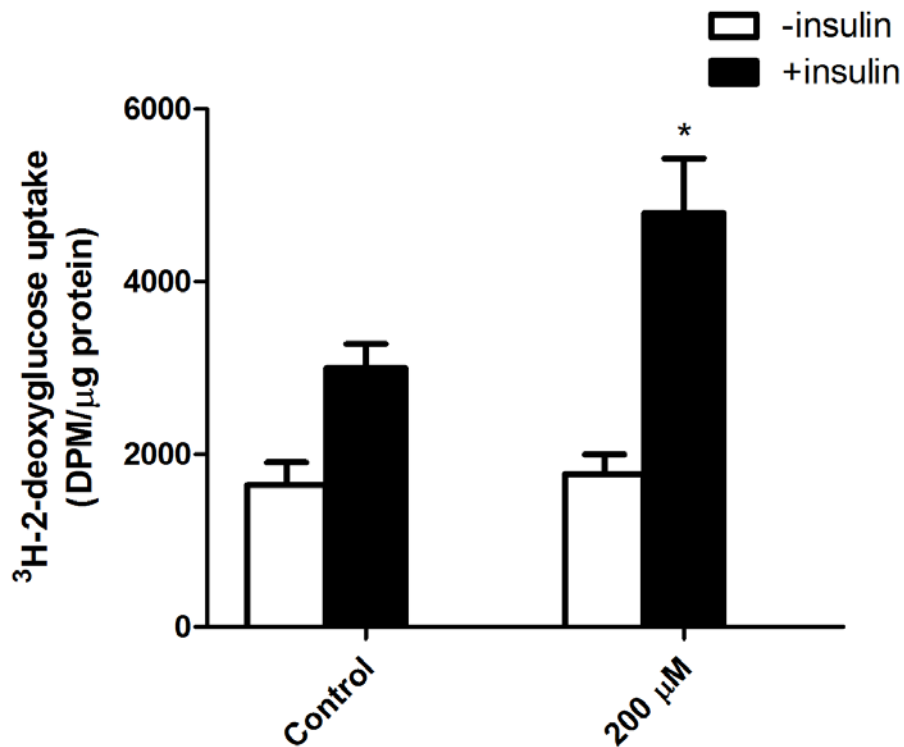
Gene		Sequence
IL-6	Forward	5'-TAG TCC TTC CTA CCC CAA TTT CC -3'
	Reverse	5'- TTG GTC CTT AGC CAC TCC TTC-3'
36B4	Forward	5'-AGA TTC GGG ATA TGC TGT TGG C-3'
	Reverse	5'-TCG GGT CCT AGA CCA GTG TTC -3'
$\beta$ -actin	Forward	5'- AGT GTG ACG TTG ACA TCC GTA-3'
	Reverse	5'-GCC AGA GCA GTA ATC TCC TTC T -3'



**Fig. 10. The effect of SDA and DHA on LPS induced IL-6 gene expression in RAW264.7 cells** (A) Cells were treated with 50  $\mu$ M SDA (A) or 200  $\mu$ M DHA (B) overnight, followed by incubation with 100 ng LPS for 8 h. mRNA expression of IL-6 was assayed by RT-PCR. Values were normalized to the level of  $\beta$ -actin mRNA. Data are presented as fold change,  $n=3$  (A) or 1 (B); different from control: \*  $P < 0.05$  (the statistical analysis was based on  $\Delta$ Ct values).

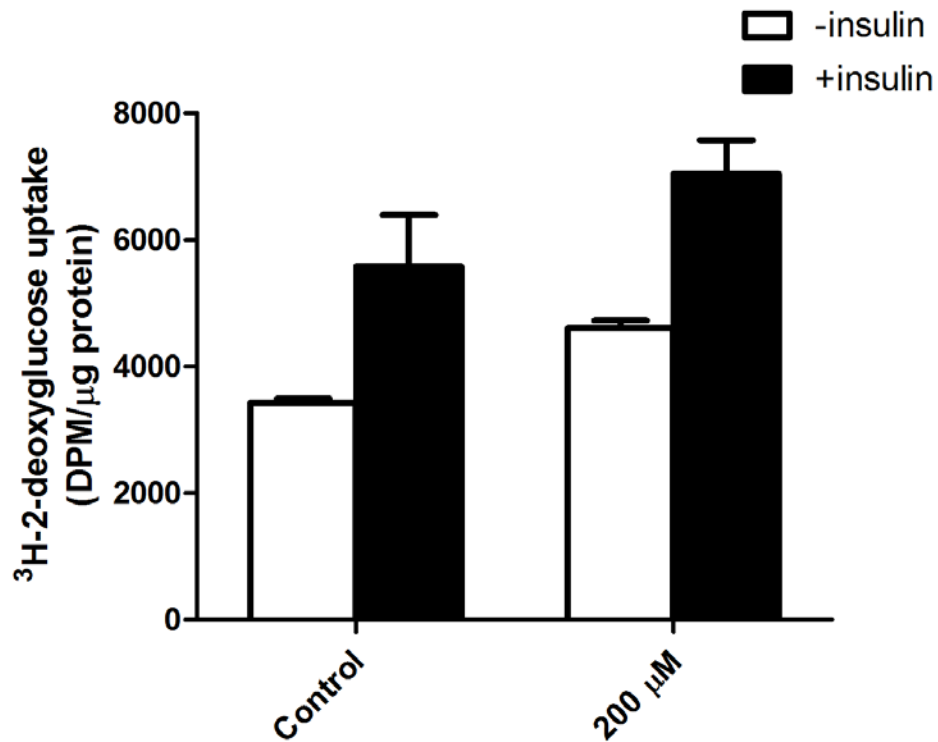


**Fig. 11. The effect of n-3 PUFA on conditioned medium-induced IL-6 gene expression in 3T3-L1 adipocytes** Day 8-12 3T3-L1 adipocytes were incubated with 200  $\mu$ M SDA, EPA, ALA overnight, followed by incubation with DMEM or macrophage-derived conditioned medium (CM) for another 24h. Adipocytes incubated with BSA vehicle followed by treatment with DMEM or CM was used as blank or control group respectively. mRNA expression of IL-6 was assayed by RT-PCR. Values were normalized to the level of 36B4 mRNA and the fold changes are represented as mean  $\pm$  SD of two independent experiments; difference from blank: #  $P < 0.05$ ; different from control: \*  $P < 0.05$  (the statistical analysis was based on  $\Delta$ Ct values).

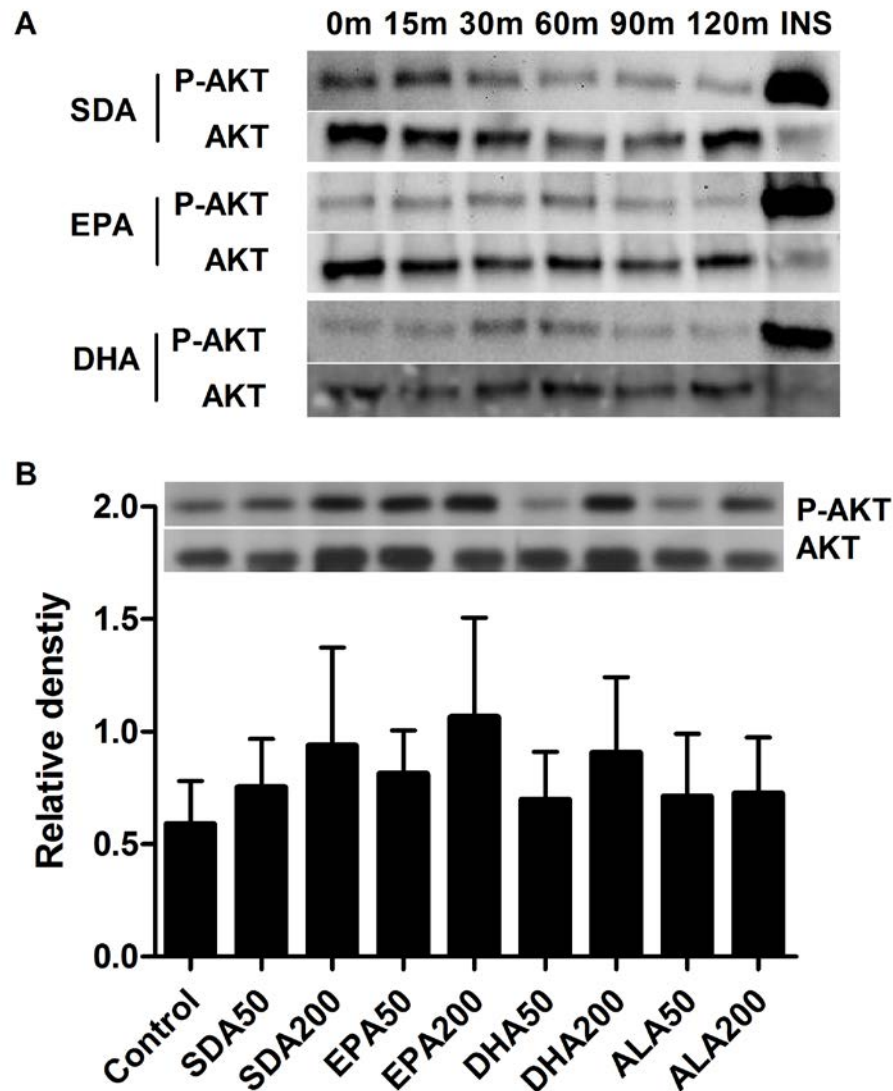


**Fig. 12. The effect of pretreatment of SDA on insulin-stimulated glucose uptake in 3T3-L1 adipocytes.** Day 8-12 3T3-L1 adipocytes in 12-well plates were pretreated with 200  $\mu$ M SDA for 24 h. After serum starved for 2 hours, cells were then stimulated with or without 100 nM insulin for 30 minutes and assayed for [ $^3$ H]-2-DOG uptake over 10 minutes. Data are mean  $\pm$  SD from two independent experiments performed in triplicate. Significant difference was determined by comparing the means of basal or insulin-stimulated 2-DOG uptake of cells treated with 200  $\mu$ M SDA to that from the BSA vehicle controls; \*  $P < 0.05$ .

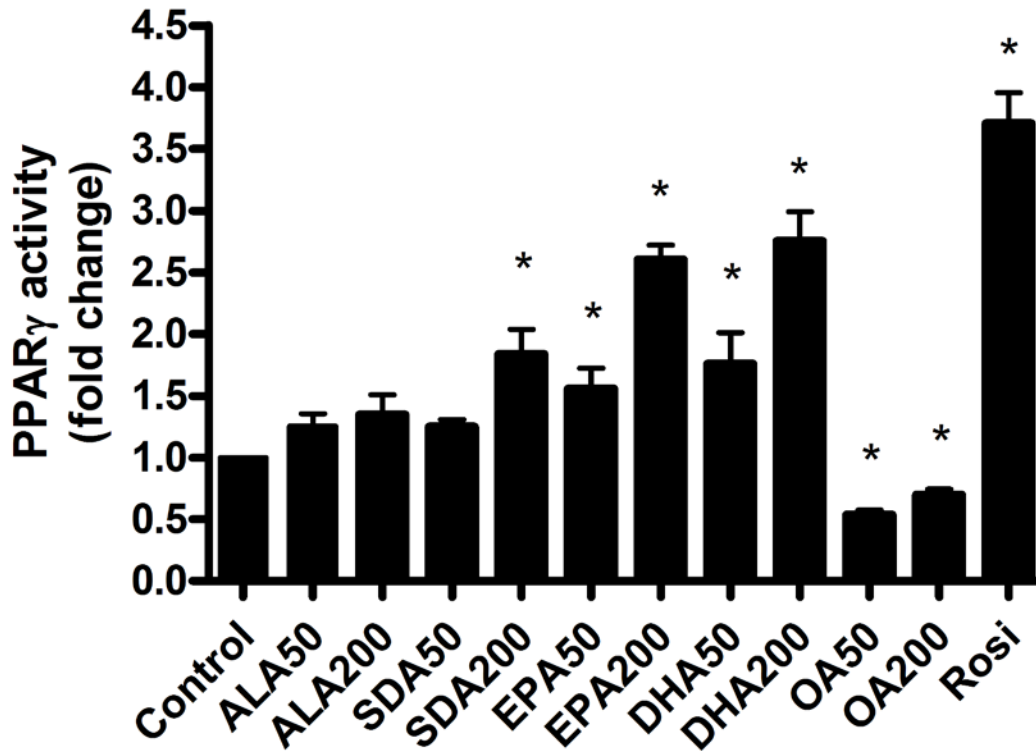




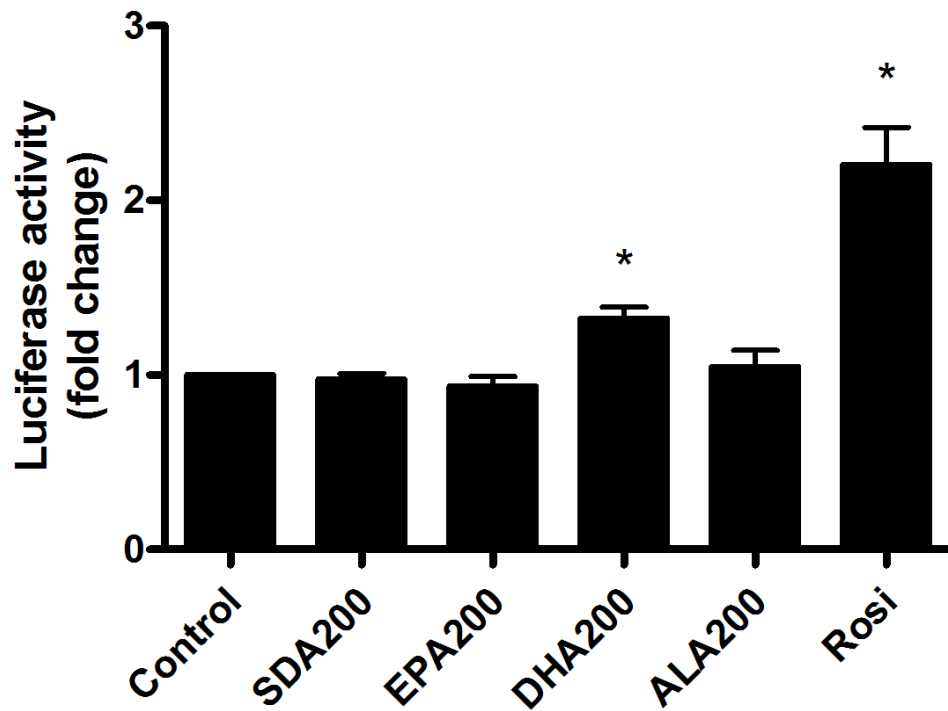
**Fig. 13. The effect of short-term SDA treatment on insulin-stimulated glucose uptake in 3T3-L1 adipocytes.** Day 8-12 3T3-L1 adipocytes in 12-well plates were serum starved for 2 h, and then pretreated for 30 minutes with 200 μM SDA or BSA vehicle separately. Cells were then stimulated with or without 100 nM insulin for 30 minutes and assayed for [<sup>3</sup>H]-2-DOG uptake over 10 minutes. Data are given as mean ± SD (n=3).



**Figure 14. The effect of n-3 PUFA on phosphorylation of AKT in 3T3-L1 cells.** (A) Day 8-12 3T3-L1 adipocytes in 6-well plates were serum starved for 4 hours, and then treated with SDA, EPA, DHA (200  $\mu$ M), BSA vehicle for different time (0, 15, 30, 60, 90 and 120 min), or insulin (INS, as a positive control) for 30min. (B) Day 8-12 3T3-L1 adipocytes were serum starved for 4 hours, and then treated with ALA, SDA, EPA, DHA (50 or 200  $\mu$ M) or vehicle for 30min. Phosphorylation of AKT was assessed by western blot analysis with anti-phospho-AKT antibody and anti-AKT antibody. Shown are representative blot and the relative expression of phospho-AKT was quantified according to total AKT level. Values were obtained from three independent and are expressed as means  $\pm$  SD; No significant difference were observed between n-3 PUFA treated cells and BSA vehicle treated control cells.



**Fig. 15. The effect of SDA on PPAR $\gamma$  activity.** HEK 293 cells were co-transfected with PPRE X3-TK-Luc, pBABE puro PPAR $\gamma$ 2 and PSV sport RXR $\alpha$ , pcDNA- $\beta$  gal. 24 h after transfection, the cells were treated with fatty acids (50 or 200  $\mu$ M), rosiglitazone (Rosi, 10  $\mu$ M) or BSA vehicle control, and luciferase activity was measured at 24 h. Luciferase activities were normalized to  $\beta$ -Galactosidase activity and are presented as the fold change of luciferase activity measured in the presence of stimulus relative to the activity of control cells with no stimulation. Values were obtained from three independent experiments performed duplicate or triplicate and are expressed as means  $\pm$  SD; different from BSA vehicle treated control cells: \*  $P < 0.05$



**Fig. 16.** The effect of n-3 PUFA on transactivation of  $\alpha$ P2 promoter by PPAR $\gamma$ . HEK 293 cells were co-transfected with  $\alpha$ P2- luciferase reporter, pBABE puro PPAR $\gamma$ 2, PSV sport RXR $\alpha$  and pcDNA- $\beta$  gal plasmid. The cells were treated with fatty acids (50 or 200  $\mu$ M), rosiglitazone (Rosi, 10  $\mu$ M) or BSA vehicle control, and luciferase activity was measured at 24 h. Luciferase activities were normalized to  $\beta$ -Galactosidase activity and are presented as the fold change of luciferase activity measured in the presence of stimulus relative to the activity of control cells with no stimulation. Values were obtained from two independent experiments and are expressed as means  $\pm$  SD; difference from BSA vehicle treated control cells: \*  $P < 0.05$

## Chapter 5 Conclusion

The increasing incidence of obesity has become a major public health concern worldwide, because it is implicated various diseases including insulin resistance, type 2 diabetes, hypertension, cancer and coronary heart disease. Consumption of fish oil containing high levels of n-3 PUFAs, such as EPA and DHA have been reported to reduce obesity and increase insulin sensitivity in rodents. However, recurring concerns regarding safety, shelf-life, palatability, and a sustainable supply of fish provide obstacles for many individuals. As such, there is a demand for alternative sources of highly unsaturated n-3 PUFA to meet the current recommendations. SDA has been targeted as a potential biologically active surrogate for EPA due to its efficient conversion after consumption. In our current study, we investigated whether SDA has potential anti-obesity, anti-inflammatory and insulin-sensitizing properties.

Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from preadipocytes. We demonstrate, for the first time, that SDA had the similar effect with DHA or EPA and was more effective than ALA on reducing the lipid accumulation in the 3T3-L1 adipocyte differentiating process. Furthermore, we demonstrated that the effects of SDA on adipocyte differentiation may work on multiple molecular targets and complex mechanisms, including inhibition of mRNA of early transcription factors C/EBP $\beta$ , PPAR $\gamma$ 2, C/EBP $\alpha$  and SREBP-1, and suppression of lipid and glucose accumulation genes SCD-1, FAS, aP2 and GLUT4 and adipocyte specific protein adiponectin. The cell proliferation assay showed that SDA treatment markedly reduced the second cycle of 3T3-L1 cell division, which suggested

that SDA may block adipocyte differentiation through a partial inhibition of 3T3-L1 cell proliferation. Thus, our study suggested that SDA may possess anti-obesity effects through prevention of adipogenesis.

Inflammation has been considered as a link between obesity and insulin resistance. The present study showed that SDA inhibited LPS-mediated inflammation in macrophage cells, and it was more effective than DHA and ALA on attenuating inflammatory cytokine expression in 3T3-L1 adipocyte exposed to macrophage-conditioned media. In addition, SDA treatment can improve insulin-stimulated glucose uptake in 3T3-L1 adipocytes. SDA also can mimic DHA and EPA to facilitate insulin signaling transduction by phosphorylation modification of AKT in 3T3-L1 adipocytes. The nuclear hormone receptor PPAR $\gamma$  has been shown to have an important role in regulating inflammation and insulin sensitivity. Our study demonstrated that SDA, EPA and DHA can increase PPAR $\gamma$  transactivity in HEK293. Thus, we proposed that the beneficial effects of SDA inflammation and insulin sensitivity may be mediated by activation of PPAR $\gamma$ .

In summary, we concluded that SDA inhibited adipocyte proliferation and differentiation, attenuated inflammation and improved insulin sensitivity in 3T3-L1 adipocyte. Although the effects of SDA on adipogenesis, inflammation and insulin sensitivity remain to be determined in vivo, our study suggested that SDA could be a botanical source of n-3 PUFA in the treatment of obesity and type 2 diabetes.

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